# Effects-Directed Analysis of Soluble Organics in Bitumen-Influenced Waters

by

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A thesis

presented to the University of Waterloo

in fulfilment of the

thesis requirement for the degree of

Master of Science

in

Biology (Water)

Waterloo, Ontario, Canada, 2019

## Authors Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

## Statement of Contributions

The organization of this thesis is such that it is composed of four chapters, two of which have been structured in a manuscript format for publication in peer-review journals. Chapter 1 is a general introductory chapter outlining information contained in the subsequent chapters. The following two chapters are in the process of submission (Chapters 2, and 3). Chapter 2 is the low-volume method development from the standardized acute *Hyalella azteca* test method for effects-directed analysis. Chapter 3 is the effects-directed analysis of bioactive polar organic fractions from industrial and natural sources. Chapter 4 is a concluding chapter summarizing the findings of this thesis. The titles and authorships of both manuscripts are listed below, along with the summary of contributions from each co-author. All chapters were written exclusively by Maegan R. Rodrigues as indicated by primary authorship.

**Chapter 2.** Maegan R. Rodrigues, A. J. Bartlett, L. M. Hewitt, D. M. Schissler, L. R. Brown, L. E. Deeth, D. G. Dixon, R. A. Frank. 2019. Development of a Low-Volume *Hyalella azteca* Toxicity Test Method for Effects-Directed Analysis.

**Contribution of co-authors:**

L. M. Hewitt, A. J. Bartlett, and R. A. Frank (Environment and Climate Change Canada, Burlington, ON) provided cadmium, potassium chloride, oil sands extracts, equipment, supplies, and aided in experimental design and method development for the miniaturized method adapted from standard testing procedures. Editorial comments and points of discussion were also provided.

L. E. Deeth (University of Guelph, ON) provided guidance and aided in statistical analysis of bioassays.

L. R. Brown and D. M. Schissler (Environment and Climate Change Canada, Burlington, ON) aided in bioassay exposures of *H. azteca*.

**Chapter 3.** Maegan R. Rodrigues, R. A. Frank, A. J. Bartlett, L. E. Deeth, M.W. Dunning, D. G. Dixon, L. M. Hewitt. 2019. Effects-directed Analysis of Bioactive Polar Organic Fractions from Industrial and Natural Sources.

**Contribution of co-authors:**

R. A. Frank provided oil sands extracts, equipment and expertise for *V. fischeri* exposures. Aided in experimental design and method development for biological analysis of dissolved organics. Editorial comments and points of discussion were also provided.

A. J. Bartlett provided equipment and expertise for *H. azteca* exposures.

L. E. Deeth provided guidance and aided in statistical analysis of bioassays.

M. W. Dunning (Environment and Climate Change Canada, Burlington, ON) provided LC-QToF analysis of dissolved organics.

L. M. Hewitt provided equipment and aided in experimental design and method development for fractionation and chemical analysis of dissolved organics. Editorial comments and points of discussion were also provided.

## Abstract

Identification of the organic compounds of concern within bitumen-influenced waters is critical for effective monitoring and reclamation within Canada's oil sands region. Effects-directed analysis (EDA) is a tool used to identify the toxic compound(s) in toxic mixtures through means of mixture fractionation, and biological and chemical analyses. Bioactive polar organic fractions (1°F3) from natural and industrial bitumen-influenced water sources (Aged oil sands process affected water (OSPW) – 1, OSPW Influenced Groundwater (GW) – 1, Natural GW – 1, and Natural GW – 2) subjected to a first tier EDA, were further fractionated in a second tier using reverse-phase high-performance liquid chromatography (HPLC). Four secondary fractions (2°F1, 2°F2, 2°F3, 2°F4), separated by polarity, were created along with a recombined treatment (2°FR) of the secondary fractions for sites OSPW influenced GW – 1, and Natural GW – 2. Toxicity was assessed using the Microtox® assay and *Hyallela azteca* due to sensitivities exhibited to the first tier EDA fraction. Due to the volume restraints of the second tier EDA analysis, a low-volume test method (50 mL and 10 organisms) was developed for the *Hyalella azteca,* which was a 4 – 8-fold volume reduction in comparison to standardized methods (200 – 400 mL and 15 – 20 organisms). Parallel tests of the low-volume method and standard method were completed to compare LC50s with two inorganic standards (KCl and $CdCl_2$) and two organic solutions (naphthenic acid fraction component (NAFC) 2009 and NAFC 2011). Finally, chemical analysis of primary and secondary fractions was completed in positive and negative electrospray ionization (ESI) mode on a liquid chromatography quadrupole time of flight mass spectrometer (LC-QToF/MS).

For the proposed *Hyalella azteca* low-volume test method, there was no evidence the LC50 values for KCl, NAFC 2009, and NAFC 2011 were different ($p = 0.34$, 0.73, and 0.48),

however, there was some evidence the LC50 values for $CdCl_2$ was different ($p = 0.037$). The percent difference between LC50 values were less than 1 – fold for KCl, NAFC 2009, and NAFC 2011, and was 2 – fold for $CdCl_2$. Due to percent differences below 3 – fold, and 95% confidence intervals (CI) which overlap, the low-volume method is determined to be an acceptable replacement for standardized methods in testing situations with access to minimal volumes of solution.

The Microtox® and reduced volume *H. azteca* assays were most sensitive to the polar organics of the primary fractions and secondary fraction recombined treatments. The toxicity response to the primary fraction was not observed in any of the individual secondary fractions, however, 2°FR from OSPW Influenced GW – 1 for the Microtox® assay (40% viability) elicited a comparable response to the 1°F3 (29% viability, $p = 0.07$). Additionally, there was no difference from Natural GW – 2 2°FR to 1°F3 (29 % viability) of the Microtox® assay (40% viability, $p = 0.054$) and the 1°F3 (0% survival) of the *H. azteca* assay (0% survival, $p = 1.00$). For the *V. fischeri* there were no obvious trends in the toxicity of the individual secondary fractions between sources but 2°F3 and 2°F4 had the greatest response within groundwater sites. Similarly, for the *H. azteca* there was no toxicological response in the individual secondary fractions, save for 2°F4 in the groundwaters: OSPW Influenced GW – 1 (78% survival), Natural GW – 1 (62% survival), and Natural GW – 2 (47% survival). The LC-QToF/MS total ion chromatograms (TICs) of the secondary fractions, were less complex in comparison to the primary fractions, but remain too chemically complex for compound identification. Additionally, the fraction profiles were distinguishable from the others with some degree of overlap between adjacent fractions.

In conclusion, the lack of recoverable toxicity in the individual secondary fractions may indicate that there are interactive effects within the more complex mixtures of polar organics

(e.g. 1°F3, 2°FR fractions) that lead to greater toxic potency than when the chemical components are separated into less complex mixtures (e.g. individual secondary fractions). Or, perhaps, the compounds driving the toxicity are split between adjacent fractions, below their response thresholds. Additionally, due to the complexity of the secondary fractions identified by the LC-QToF, another tier of EDA is required to analyze unknowns. It is therefore recommended that this second tier of EDA must be reinvestigated such that the secondary fractions are combined with their adjacent fraction.

**Acknowledgments**

First and foremost, I would like to thank my parents for supporting me as I pursued my schooling in science. Thank you for helping me navigate the emotional ups and downs of my academic career, and for always being my biggest supporters.

To my supervisor George Dixon, thank you for taking a chance on me and providing me with this amazing researching opportunity. To my co-supervisors Richard Frank, and Mark Hewitt, I could never thank you enough for all the guidance provided. You two have had the patience and understanding which has allowed me to grow as an individual over these past two years.

I also would like to thank my team at Environment Canada: Michael Dunning, Sheena Campbell, Maddy Cosme, Martina Rudy, Ruth Vanderveen and past members Daniel Schissler and Katie McCann. Without your help, support, and free coffee, I would not be where I am today. Additional thanks to the Environment Canada *Hyalella* group, Adrienne Bartlett, Lisa Brown, and Amanda Hedges, for allowing me to share your space, knowledge, and recipes.

To my friends, you know who you are, thank you for your shared understanding and experiences.

Finally, I would like to thank my partner Samuel Aylward for his constant love and support. Without you these past two years would have been infinitely harder and less fun.

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## List of Abbreviations

1°F3: primary polar organic fraction three

2°F: Secondary fraction

AEO: acid extractable organic

ANOVA: analysis of variance

$CdCl_2$: cadmium

CI: confidence interval

DO: dissolved oxygen

EDA: effects-directed analysis

ESI: Electrospray ionization mode

$H_2O$: water

HCl: hydrochloric acid

HPLC: high performance liquid chromatography

KCl: potassium chloride

LC50: median lethal concentration

LC-QToF: liquid chromatography quadrupole time-of-flight mass spectrometry

MeOH: methanol

NA: naphthenic acid

NAFC: naphthenic acid fraction component

OSPW: Oil Sands Process-affected Waters

SPE: Solid-phase extraction

TIC: Total ion chromatograms

WWE: Whole water equivalents

# Chapter 1 : Introduction

## 1.1 Introduction

The Canadian oil sands region, primarily located in northern Alberta, is one of the largest crude oil deposits in the world with established recoverable reserves of 174 billion barrels of bitumen and crude oil (Alberta Energy and Utilities Board, 2016-2017; Allen, 2008). Bitumen is a mixture of viscous hydrocarbons which when broken down becomes a sought-after resource by many countries. The Alberta oil sands region encompasses three deposits: Peace River, Cold Lake, and the Athabasca. The Athabasca deposit is the largest and contains 2700 $km^2$ of surface mineable bitumen (e.g. oil sand deposit < 75m below surface). Cold Lake, the second largest deposit, and Peace River, both contain deposits which are unrecoverable by surface mining practices; these may be extracted via in-situ processes.

The extraction and production of bitumen has rapidly expanded in the past few decades, with 2.83 million barrels of bitumen recovered per day in 2017 (Alberta Energy and Utilities Board, 2017 - 2018). The extraction processes require a significant volume of water which is collected from the Athabasca River watershed and is recycled. Two major extraction methods exist for the removal of bitumen from the encompassing sands; surface mining and *in situ* methods (for materials greater than 75 m below the surface). An adaption of the Clark extraction is used for surface mining of bitumen such that excavated materials are mixed with a caustic solution of hot water, which separates the bitumen from surrounding sand and clay (FTFC, 1995a). Once the bitumen is separated, the bitumen is skimmed from the solution's surface (Allen, 2008; Shah et al., 2010). The practice of bitumen extraction requires 2 - 4.5 barrels of water per 1 barrel of crude oil collected (Alberta Energy and Utilities Board, 2016-2017; National Energy Board, 2015). Comparatively, *in situ* methods recover bitumen at depths beyond those capable in surface mining (>75 m) and require approximately 1 barrel of water per barrel of crude oil. An example of a commonly used *in situ* method is steam assisted gravity drainage.

Two existing wells are utilized for this method, in which steam is injected into the highest of the two wells. The heat from the steam reduces the viscosity of the oil in the surrounding area allowing it to drain into a lower well. Once enough oil has collected into the lower well it is pumped to the surface for further processing (Shah et al., 2010).

Although each process requires significant water usage for bitumen extraction, approximately 95% of the oil sands process-affected water (OSPW) used is recycled. However, water reuse is limited by accumulations of dissolved salts and minerals, trace metals, soluble organics, and overall alkalinity (Allen, 2008). After the extraction process, OSPW, also known as fluid tailings, is pumped into tailings ponds where suspended solids settle over time. Tailings ponds are large settling basins that are used to collect solid tailings and store fluid tailings for reuse (FTFC, 1995b). Currently, there are over 98 $km^2$ of fluid tailings in these ponds across Alberta (Government of Alberta, 2018). Owing to the active recycling of OSPW, the concentrations of extraction materials are elevated in end of life OSPW and tailings ponds (Allen, 2008; Brown & Ulrich, 2015). Additionally, both fresh and end of life OSPW and tailings materials are acutely toxic to aquatic biota (Anderson et al., 2012; Frank et al., 2009; Goff et al., 2013; Hagen et al., 2014; He et al., 2012; Kavanagh et al., 2013; Marentette et al., 2015a). The toxicity is attributed to metals, ammonia, and soluble organic compounds (including naphthenic acids (NAs) and other acid extractable organics (AEO)) all of which are released during bitumen extraction (Allen, 2008; MacKinnon & Sethi, 1993). Due to the toxic nature of OSPW and tailings wastes (such as ore and effluent left from bitumen extraction), the Government of Alberta has an established zero discharge policy in which there are no permits issued for the discharge of untreated wastes (Allen, 2008; Government Of Alberta, 2017). Additionally, the government of Alberta requires industries to remediate and reclaim 100 percent of the land used for bitumen extraction (Government Of Alberta, 2017). Industry has used

reclamation approaches that involve capping OSPW tailings pond wastes, which are stored in large empty mining pits, with water to create end-pit lakes (Langseth et al., 2015). However, due to the toxicity associated with OSPW, there is concern that contaminants will seep from the pond into adjacent groundwaters and surface waters. This concern has been verified by studies which provide evidence of seepage from these containments (Ferguson et al., 2009; Frank et al., 2014; Hewitt et al., 2019; Milestone et al., 2019; Ross et al., 2012). A major concern and requirement for reclamation is the determination of toxic drivers from these tailings ponds that could potentially have long term adverse effects on the natural biota and environment. Additionally, knowledge of the compounds causing toxicity can help create water quality guidelines with effective monitoring applications. However, research into OSPW toxicity is complex and has been studied for many years. Efforts are still being made to understand these complex mixtures and source pathways in the environment so that relevant water quality guidelines and reference materials can be established. Challenges arise in pinpointing significant compounds due to the lack of consistency in these mixtures (spatial and temporal) upon testing and variability in analytical methods.

### 1.1.2 Polar Organic Compounds of Oil Sands Process-Affected Waters

A class of compounds significant to the water-soluble organic component of bitumen and tailings OSPW is the mixture of polar organics. These organic compounds are dispersed within bitumen deposits leading to high concentrations within tailings after extraction (Clemente & Fedorak, 2005). However, organic mixtures within OSPW and other bitumen-influenced waters are quite complex such that the compounds in these mixtures are dependent on factors which include pond age, depth and location of source materials, and proximity to tailings seepage passing through tailings containments (Frank et al., 2016).

Acid-extractable organics (AEOs) are a sub-group of compounds which largely contribute to polar organic mixtures within OSPW. Initially naphthenic acids (NAs), which are estimated to account for less than 50 % by mass of the organic compounds found in OSPW (Grewer et al., 2010; Headley et al., 2009), were thought to be the polar organic compounds of concern. However, the term NA is misleading with respect to OSPW, due to the complex mixture of acid-extractable organics present (Grewer et al., 2010) and known differences between commercial NAs derived from petroleum (Marentette et al., 2015a).

Naphthenic acids (NAs) are classically defined as a mixture of alkyl-substituted acyclic-aliphatic and cycloaliphatic carboxylic acids. The formula for classical NAs is $C_nH_{2n+z}O_2$, where n is the number of carbon atoms, and z is either 0 or a negative even integer denoting the unsaturation of the structure due to the inclusion of ring structures (Clemente & Fedorak, 2005). The components of NAs exhibit properties of both hydrophobic (aliphatic side chain) and hydrophilic (polar carboxylic group) nature which characterises them as surfactants (Brient et al., 1995). Combining their concentrations and amphiphilic properties, these compounds have been the focus of toxicological concern. Despite the focus on classical NAs within tailings, attention has shifted to AEOs because NAs are one compound class within the broader and complex AEO mixtures. As well, past attempts at characterizing OSPW AEOs have led to the discovery of new compounds beyond the classical NA definition (Grewer et al., 2010; Rowland et al., 2014; Rowland et al., 2011a; Rowland et al., 2011b; Rowland et al., 2011c; Wilde et al., 2015). Due to the complexity of polar organics found in crude oil deposits both AEOs and NAs are largely uncharacterized, and the toxicity of these water-soluble organics remains poorly understood.

The difficulty associated with the elucidation of compound classes in OSPW presents challenges for the accuracy and specificity of traditional instrumentation (i.e., LC/MS, GC/MS, etc.) to characterize the components within. As such, there has been a shift to high or ultra-high-

resolution mass spectrometry instrumentation, and instrumentation with enhanced chromatographic separations (e.g. two-dimensional GC), for these applications. The high-resolution instrumentation provides greater mass accuracy and can thus achieve greater resolution of the mixtures of AEOs (Brunswick et al., 2015). Once applied, this technology can be used to characterize the compound classes and determine new compound structures within AEO mixtures. Previous analysis of OSPW extracts discovered evidence of dicarboxylic acids (Rowland et al., 2011b; Rowland et al., 2011c), species containing nitrogen and sulphur (Rowland et al., 2014), diamondoid tricyclic acids (Rowland et al., 2011a), adamantane carboxylic acids, and adamantane ethanoic acid (Rowland et al., 2011a; Rowland et al., 2011c). Additionally, nearly 30 types of alicyclic and bicyclic compounds have been identified in OSPW, many of the compounds containing stereoisomers (Wilde et al., 2015). Despite these advances, the complexities of the soluble organics remain a challenge to elucidate due to the multitude of compound classes present.

### 1.1.3: Effects-directed Analysis

Knowledge of the effects of bitumen-influenced waters on aquatic environments has been expanding for quite some time along with the knowledge that these aquatic ecosystems are often sinks for industrial and natural contaminants. The overall effect of this widespread contamination is often difficult to measure and understand. Effects-directed analysis (EDA) is an approach that can be used to identify the drivers of toxicity within complex mixtures of concern (Brack et al., 2016). Once a sample has been identified as being toxic, EDA utilizes a series of steps to identify the principal toxic components within complex mixtures using iterative steps of chemical fractionation guided by bioassay results. Figure 1.1 further describes the steps EDA uses as an approach to isolate and characterize toxicants.

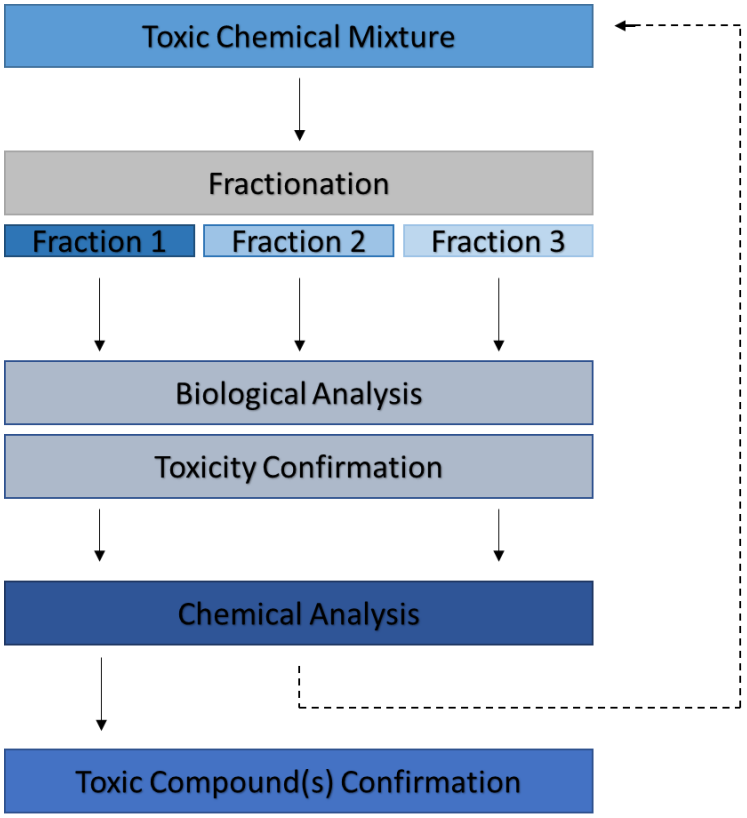


**Figure 1-1:** Generic Scheme of EDA (Brack et al., 2016).

The source material (i.e., toxic water sample) is typically a complex mixture and therefore must be fractionated before use in bioassays, using one of many techniques such as chromatography, etc. This ensures that EDA can focus solely on bioactive chemicals. However, the separation and characterization of single compounds within bitumen-influenced waters is often challenging and will require many steps due to the complexity of the mixtures.

Owing to its toxicity, OSPW has been investigated using EDA approaches. Previous methods have fractionated OSPW based on: empirical formula class (Morandi et al., 2015), pH (Bauer et al., 2015), solubility (Grbovic et al., 2012), aromaticity (Jones et al., 2012), microbial biodegradation (Toor et al., 2013), and distillation properties (Frank et al., 2008). These techniques have helped to simplify and identify mixture components of the waters, but the ability

to achieve chemically distinct fractions has remained elusive. Furthermore, many studies have investigated commercial naphthenic acid mixtures which are considerably different from OSPW and therefore are ineffective at determining the organic compounds responsible for OSPW toxicity (Bartlett et al., 2017; Marentette et al., 2015a).

Analytical separation technologies and EDA have been used to assess the acute toxicity of OSPW dissolved organic chemical classes on fathead minnows (*Pimephales promelas*) and Microtox® (Morandi et al., 2015). More specifically Morandi et al. (2015) fractionated OSPW based on empirical formula class, using a reverse-phased HPLC and hybrid linear ion trap-Orbitrap mass spectrometer (Morandi et al., 2015). This method is much more specific and involved in comparison to the Bauer et al. (2019) solid phase extraction method. The Bauer et al. (2019) method fractionated samples of an aged tailings water and bitumen-influenced groundwaters into three fractions based on polarity and pH (Bauer, 2018; Bauer et al., 2019). Toxicity testing of these fractions with a suite of aquatic organisms revealed the most polar, and least polar, soluble organics to be the most bioactive fractions (Figure 1-2). These toxic fractions require further investigation using an effects-based approach.

### 1.1.4: Toxicity Testing of Bitumen-Influenced Waters and Toxicants

Fresh OSPW fractions have proven to be acutely toxic to aquatic organisms with considerable studies attributing toxicity to surfactants and polar organic compounds (Gagne et al., 2011; Marentette et al., 2015a; Marentette et al., 2015b; Morandi et al., 2015; Scarlett et al., 2013). Studies found both classical and non-classical NAs, and polar organic compounds, to be toxic to microorganisms, aquatic algae, fish, invertebrates, vegetation, birds, and mammals (Bartlett et al., 2017; Headley & McMartin, 2004; Kindzierski & Jin, 2012; Marentette et al., 2015a).

As stated earlier, the identification of the polar organic compounds, including AEOs, is a complex problem because each bitumen-influenced water source contains a different and distinct mixture of soluble organic compounds (Armstrong et al., 2009; Clemente et al., 2003; Grewer et al., 2010). To combat these issues, there have been a number of studies which related differing physio-chemical properties to AEO acute toxicity (Bauer, 2018). The properties include AEO molecular weight (Clemente & Fedorak, 2004; Frank et al., 2008), solubility (Jones et al., 2011; Stanford et al., 2007), carboxylic acid content (Frank et al., 2009), aromaticity (Jones et al., 2011), and number of carbons (Lai et al., 1996). Due to the inability to pinpoint exact structures of all OSPW toxic compounds, some assumptions can be made. As a result of the surfactant properties of NAs and other organic acid compounds, it is likely that narcosis, a non-specific mode of action, will occur (Frank et al., 2009). In such cases of narcosis, when a hydrophobic molecule enters the lipid bilayer in a cell, it causes membrane disruption which in turns affects the membrane properties and can lead to cell death (Frank et al., 2009; Klopman et al., 1999; Konemann, 1981). Additionally, prior studies lead to the attribution of toxicity within OSPW to a decrease in molecular weight. These studies found that tailings aged 1 to 24 months have decreasing acute toxicity (MacKinnon & Boerger, 1986) and additional studies found 4 to 6 week old OSPW is decreasingly toxic to fathead minnows as compared to fresh OSPW (Lai et al., 1996). The decreasing toxicity of aged tailings was credited to the biodegradation of NAs with less carbon molecules, and lower molecular weight, by indigenous microbes (Biryukova et al., 2007; Clemente & Fedorak, 2004). It was presumed that classical NAs of low molecular weight were more toxic than NAs of large molecular weight due to bioavailability and ability to traverse cellular membranes. However, recent work found no definitive trend between molecular weight and toxicity when comparing sensitivities of multiple fish species (Bauer et al., 2017).

### 1.2 Bulk Extraction of Bitumen-influenced Waters and Resulting Toxicity

A recently developed fractionation technique (Bauer, 2018; Bauer et al., 2019) attempted to assess the toxic potential of dissolved organic compounds in OSPW and naturally occurring bitumen-influenced waters through an EDA approach (Bauer, 2018; Bauer et al., 2019; Frank et al., 2019a). This study created a preparative-scale extraction and fractionation technique separating bitumen-influenced waters via differences in polarity using reverse phased Solid Phase Extraction (SPE). The novelty of this method is that it can be applied to all bitumen-influenced waters with a degree of reproducibility in large volumes. The creation of large quantities of fractions is beneficial for use in multiple toxicity applications, chemical analysis, and as a platform for EDA studies.

Initial efforts with this method focused on an aged tailings sample that is relevant to the conditions of end-pit lakes and other bitumen-influenced waters influenced by natural degradation processes. The method was then applied to other bitumen-influenced waters that included groundwater from sites containing only natural bitumen input (≥1 km from OSPW sources) as well as, OSPW influences (≤200 m of a known OSPW source). These sites were previously identified as containing natural bitumen compounds, or OSPW influence due to OSPW seepage in groundwaters near to OSPW containments (Bauer et al., 2019; Frank et al., 2014; Hewitt et al., 2019; Milestone et al., 2019). More recently the similarities between chemical profiles and toxicity between natural and mixed OSPW groundwaters have become apparent (Bauer et al., 2019; Frank et al., 2018) however additional testing is required.

The bitumen-influenced waters were fractionated into three increasingly polar fractions: low polarity, intermediate polarity, and high polarity organics (Bauer, 2018; Bauer et al., 2019). The total volume of fraction extract collected from each source water was recorded and equated

to the original volumes of the whole waters (x mL of fraction is equivalent to 1 L of source water) to create the whole water equivalents (WWE).

**Table 1-1:** Whole water equivalents (WWE) of bitumen-influenced water sites primary polar organic fraction based on extraction volumes from the bulk solid phase extraction method.

| Site | 1 L equivalents [mL] |
|---|---|
| Aged OSPW - 1 | 17 |
| OSPW Influenced GW - 1 | 22 |
| Natural GW - 1 | 23 |
| Natural GW - 2 | 30 |

Bioassay fraction volumes were prepared according to the WWE of the fractions for each site (Bauer, 2018). Aquatic organisms from multiple taxa were exposed to the three fractions, whole waters, and a recombined treatment. Acute assays were run with *Pimephales promelas, Oryzia latipes, Vibrio fischeri* (Microtox® assay), *Daphnia magna, Ceriodaphnia dubia, Hyalella azteca, Lampsilis cardoi,* and *Hexagenia* spp, providing an extensive toxicological characterization of the fractions. *H. azteca* was the most sensitive invertebrate to the dissolved organic fractions and whole waters, and toxicity was isolated to two fractions: the most and least polar (Bauer, 2018). Chemical analysis revealed the majority of classical $O_2$ NAs to be within the least polar fraction, indicating that the toxicity of dissolved organics within OSPW is not completely attributed to classical NAs. Additionally, Bauer's study found the polar organic fraction to contain a smaller abundance of $O_2$ compounds and thus elevated $O_{2+}$ content in comparison to the other fractions. Of interest, profile comparisons between natural bitumen-influenced sites and OSPW influenced groundwaters found many similarities and did not find elicit significant differences in toxic responses between natural and OSPW groundwater sites

(Bauer, 2018). However, the polar organics of aged OSPW contained a larger proportion of polyoxygenated compounds in comparison to the groundwaters (Bauer et al., 2019).

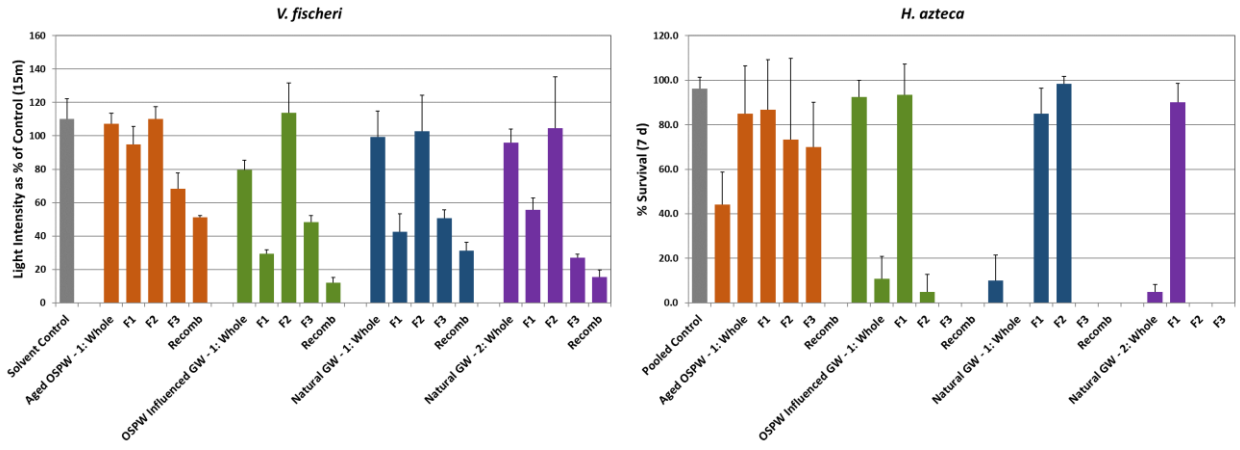


**Figure 1-2***:* Percentage viability/survival of *Vibrio fischeri* and *Hyalella azteca* (mean ± standard deviation) exposed to control water, solvent control, whole source waters (whole), primary fractions (F1 – F3), and a recombined treatment (Recomb, fractions F1 – F3 combined). Aged OSPW – 1 (orange), OSPW Influenced GW – 1 (green), Natural GW – 1 (blue), and Natural GW – 2 (purple) (Bauer et al., 2019; Frank et al., 2019a).

Additional acute and chronic toxicology data are required to understand what substances are driving toxicity in these complex mixtures of bitumen organics. Due to the lack of knowledge regarding the toxic bitumen-influenced compounds, the development of scientifically defensible environmental regulations for reclamation cannot be established. The Government of Alberta has set industrial reclamation guidelines in which 100 percent of land use must be returned to it's original state and fluid tailings must be reclaimed 10 years after the end of a mining project (Government of Alberta, 2018).

### 1.3 Research Objectives and Experiments

The objective of this thesis was to further the understanding of the toxicity and chemical composition of the dissolved organic compounds within bitumen-influenced waters; most notably between natural bitumen-influenced groundwaters and OSPW influenced groundwaters. Groundwaters were chosen because they are more concentrated in comparison to surface waters and thus the soluble organics are measurable. This study builds on the work by Bauer (2018) by applying a new tier of EDA to the previously determined toxic fractions identified by the eight organisms exposed to the fraction treatments. *Hyalella azteca* exhibited the highest sensitivity among tested invertebrate species to the dissolved organic fractions (Bauer et al., 2019; Frank et al., 2019b). Additionally, the responses of *Vibrio fischeri* to the isolated fractions and source water at 3-fold whole water equivalents (WWE) were comparable to the survival responses of the *H. azteca*. The polar organic materials of fraction three (1°F3) were chosen to continue an effects-directed analysis for multiple reasons. Firstly, it is expected that the highly polar organic compounds will have a better response to reverse-phase HPLC fractionation methods. Extremely polar compounds will likely be eluted from the column first and compounds which are less hydrophilic will elute off the column as the gradient of the less-polar mobile phase increases. Secondly, the analysis of 1°F3 found it to contain greater abundances of $O_{2+}$ containing species that were found to exhibit toxicity at lower threshold concentrations in comparison to the classical NA $O_2$ compounds contained in 1°F1 (Bauer, 2018). Furthermore, further fractionation of the primary fraction, 1°F3, is required as it is still too complex and therefore the toxicity cannot yet be attributed to specific chemical classes, such as polyoxygenated species.

Applying the next tier of EDA to the bioactive soluble polar organic fraction will decrease the complexity of the mixture and aid in the continued investigation of toxic drivers and mechanism(s) of toxicity. Simplifying the mixture through further fractionations and following

with non-targeted qualitative chemical characterization may eventually aid in the discoveries of the toxic compounds. As a result of identification, new remediation strategies aimed at the target organic compounds of interest can be implemented. For example, new water quality guidelines can set acceptable limits of the toxic compound(s), or compound classes, so that they can be monitored and regulated by outside organizations and amended by removal processes in place for industry. However, the future of environmental monitoring programs requires comparisons of OSPW and base levels of the harmful compounds in the natural waters to determine what concentration of the toxic compounds is elevated in comparison to the natural background.

### 1.3.1 Experiment 1

*Hyalella azteca*, a freshwater amphipod relevant to the Athabasca watershed, was amongst the most sensitive to the dissolved organics from the polar fraction and therefore further toxicological analysis involving this fraction would benefit from the use of this organism. However, an acute water only exposure requires volumes of 200-400 mL per replicate (i.e., 600 – 800 mL for the minimum recommended number of 3-4 replicates) (Borgmann et al., 2005a; Environment Canada, 2013) which is excessive for many EDA experiments. Due to potential limits to available materials in effects-based work, toxicity assays which require minimal materials for analysis are highly valuable. The bulk SPE fractionation method developed by Bauer et al. (2019) utilizes 180 L of bitumen-influenced waters in order to create enough materials required for full acute toxicity analysis on a variety of organisms. However, as with all EDA analyses, the availability of materials decreases with every additional tier due to overall resources and time. Therefore, a proposed low-volume acute testing method is developed which uses 50 mL of solution per replicated, or 150 mL of solution per one test.

**Objective**: To create a reduced volume biological test method for acute *H. azteca* water-only exposures.

**Hypothesis**: The LC50s (lethal concentration, 50%) from the proposed low-volume tests will be comparable to the LC50s of the full volume tests.

**Methodology**: The low-volume method under investigation utilized a volume of 50 mL and 10 juvenile organisms per test replicate. A total of 4 reference toxicants were used to test the robustness of the new methodology: 2 inorganic compounds and 2 organic mixtures. Cadmium chloride ($CdCl_2$) and potassium chloride (KCl) were the two inorganic reference toxicants which are commonly used for *H. azteca* acute bioassays. Naphthenic acid extracts from fresh tailings in 2009 and 2011 were previously analyzed (Bartlett et al., 2017) and were the two organic mixtures used as organic reference toxicants. New test methods closely mimicked standard test protocols used by Environment Canada (2013); standard tests were run concurrently for ease of comparison.

### 1.3.2 Experiment 2

Prior EDA on dissolved organics isolated from the oil sands region found bioactivity in a highly polar acid extractable organic fraction, 1°F3 (Bauer et al., 2019). Due to this, a second tier of EDA was applied to this fraction to investigate the identities of the toxic substances in naturally influenced and mixed OSPW influenced groundwaters. Continued exploration of bitumen-influenced AEOs by the sub-fractionation of the polar organic primary fraction (1°F3) will further the understanding of chemical composition via qualitative analysis and organism's sensitivities to these organics.

**Objective**: To compare toxicity and chemical composition of industry and natural polar organic AEOs in bitumen-influenced groundwater sources via sub-fractionation of 1°F3 using a new HPLC method.

**Hypothesis**: Chemically distinct and simplified secondary fractions will have varying degrees of toxicity response in comparison to the unfractionated 1°F3.

**Methodology**: The polar primary fraction (1°F3) was sub-fractionated using a new polar separation method by HPLC. Toxicity testing of the secondary fractions was completed using two species known to be sensitive to 1°F3: *Vibrio fischeri* (Microtox® assay) and *Hyalella azteca*. Microtox testing was completed at 3-fold whole water equivalents, similar to the previous analyses. The new low-volume test method was utilized for *H. azteca* tests. Toxicological test organisms were exposed to the primary fraction, secondary fractions, and a recombined treatment reassembling the secondary fractions. Additionally, the secondary fractions were chemically analyzed using the LC-QToF/MS in ESI positive and negative ion mode.

# Chapter 2 : Development of a Low-Volume *Hyalella azteca* Toxicity Test Method for Effects-Directed Analysis

## 2.1 Summary

Biological assays can be used to measure relevant toxic effects of single compounds as well as complex organic mixtures in the environment and are used with effects-directed analysis (EDA). EDA utilizes a series of steps to identify the principal toxic components within a complex mixture using iterative steps of chemical fractionation guided by bioassay results. Limitations to effects-directed assessments can be related to the volume requirements for whole organism biological analysis, and the capacity to assess multiple biological endpoints. *Hyalella azteca* is a valuable organism for biological testing of oil sands bitumen-influenced waters because it is native to the Athabasca watershed and it is sensitive to the soluble organics from these waters. The creation of a reduced volume acute toxicity test for *H. azteca* is beneficial for analyses which require this sensitive organism for toxicological endpoint determination and have limited treatment solutions available. The new low-volume method alters a static, 7-day standardized test exposure of 400 mL of test solution and 15 organisms, to use 50 mL of test solution and 10 organisms per replicate. Standard and low-volume tests with inorganic reference material toxicants (KCl and $CdCl_2$), and organic mixture solutions (two mixtures of Naphthenic acid fraction components (NAFC 2009, and NAFC 2011) were run concurrently. The LC50s from the low-volume and standardized tests were compared to upper and lower LC50 confidence intervals from the other test, and by performing a t-test statistic on the LC50 ratio. There was no difference between the LC50s of the low-volume and standardized tests for KCl, NAFC 2009, and NAFC 2011 ($p > 0.05$). The $CdCl_2$ LC50s for low-volume and standardized test methods were statistically different ($p = 0.03$). The difference between the LC50s was less than 2-fold for each toxicant, the 95% confidence intervals overlapped, and values were within expected range for the Bartlett lab, which provided strong evidence that the LC50s were not biologically different between test methods. Overall, the effectiveness of this newly developed low-volume

test method is comparable to standardized test methods and has been validated for further use in situations with limited testing materials.

## 2.2 Introduction

Effects-directed analysis (EDA) is a tool used to determine the drivers of toxicity within complex mixtures through the use of fractionation techniques and aquatic bioassays (Brack et al., 2016). Often the toxicity analysis of complex mixtures creates more questions about the toxic drivers within the mixture. For example, toxicity could be caused by an individual compound, a class of compounds, or the result of the combination of compounds. Due to the complexity of environmental mixtures, multiple tiers of EDA and biological testing are often required before the toxic components in a mixture can be determined. Owing to the consumption of the environmental samples with application of EDA, decreased volumes of the final testing solutions are attainable with each successive tier of EDA. For this reason, the number of measurable endpoints and choice of a bioassay are restricted to tests which require minimal volumes. Ideally, bioassay selection is based on environmental relevance and organism sensitivities, but volume requirements must be taken into consideration. For example, the marine bacterium *Vibrio fischeri*, (Microtox® assay), requires under 10 mL of solution to complete a 15-minute bioluminescence exposure. The volume requirements of the Microtox® assay are minimal compared to the 96 h rainbow trout standard acute toxicity test which requires 1 L of volume for every 0.5 gram of fish. A minimum of 10 fish must be selected per test, which range in size from 0.3 – 2.5 g, thus 6 L of test solutions is the lowest volume required (Environment Canada, 1990). Consequently, *Vibrio fischeri* is commonly used as a testing organism for EDA. However, caution must be used when extrapolating Microtox® assay results to fresh-water ecosystems because it uses a marine organism.

*Hyalella azteca*, a freshwater amphipod, is commonly found in many aquatic environments such as temperate lakes, ponds, slow-flowing streams, and rivers, within North America. They have been used in many acute toxicity tests with various chemicals and have proven to be a sensitive freshwater species (Bauer et al., 2019; Borgmann, 2002; Borgmann et al., 2005b; Borgmann & Munawar, 1989; Environment Canada, 2013; Phipps et al., 1995; Schubauer-Berigan et al., 1993). Due to their ubiquity and abundance, low level in food chains, and sensitivity to a wide variety of chemicals, they are an ideal organism for aquatic testing of environmental mixtures but may not be selected in EDA research due to volume requirements. Currently, standardized acute water-only *H. azteca* tests require volumes of 200 mL or more and 15 – 20 organisms per test replicate (Borgmann et al., 2005a; Environment Canada, 2013). Therefore, development of a reduced volume acute test for *H. azteca* would be beneficial for EDA applications.

However, reducing the volume of treatment solution per organism can contribute to undesirable changes in the water chemistry of the test solutions. For example, when there is minimal solution available per test organism the oxygen availability will decrease per organism, and the concentration of ammonia can increase due to degraded food and wastes (Environment Canada, 2013). Although *H. azteca* can survive exposure to low oxygen environments for extended periods of time, increased concentrations of ammonia can be detrimental to their survival (Environment Canada, 2013). Therefore, water chemistry is monitored to ensure observed responses are due to treatment exposures. Additionally, the low-volume test methods must meet the recommended test validity criterion of greater than 90 % survival in negative controls for 96-hour water-only tests (Environment Canada, 2013).

To assess the validity of this reduced volume test method, adapted from the standard Environment Canada (2013) test, parallel tests were conducted using standard methods (200 mL

and 15 amphipods per replicate) and a low-volume method (50 mL and 10 amphipods per replicate) with two inorganic compounds commonly used as reference toxicants (potassium chloride (KCl), and cadmium chloride ($CdCl_2$)), and two previously tested organic mixtures demonstrated to be toxic to *H. azteca* (naphthenic acid fraction components (NAFC) from a fresh OSPW source collected in 2009, and NAFC from 2011; Bartlett et al., 2017). The LC50s from this new low-volume acute method were compared to the parallel standard volume tests as well as the LC50s from previous studies with NAFCs (Bartlett et al., 2017).

## 2.3 Materials and Methods

### 2.3.1 Standardized Method

Static 7-day, water only *H. azteca* acute toxicity tests were conducted based on standardized test methods (Environment Canada, 2013). Juvenile amphipods used in tests were 2 - 10 days of age at test initiation. Standard and reduced volume tests were completed with two inorganic reference toxicants and two organic mixtures (Bartlett et al., 2017). The two inorganic toxicants were cadmium chloride ($CdCl_2$) and potassium chloride (KCl), and the two organic solutions were naphthenic acid extractions of fresh tailings, NAFC 2009 and NAFC 2011. These organic mixtures have been previously tested with *H. azteca*, with reported LC50's of 16.7 mg/L and 25 mg/L, respectively (Bartlett et al., 2017). A 55 % dilution series (0.25, 0.14, 0.08, 0.045, 0.025, 0.014, 0.008, 0 μM) was used for $CdCl_2$ test solutions, 80 % dilution series (0.64, 0.51, 0.41, 0.33, 0.26, 0.2, 0.16, 0 g/L) for KCl test solutions, and 50% (100, 50, 20, 10, 5, 2, 0 mg/L) dilution series used for the NAFC solutions.

Stock solutions of KCl (1 g/L) and $CdCl_2$ (0.1 mM) were prepared in Milli-Q water 24 hour prior to test set up. Stock solutions were mixed with dechlorinated water 2 hours prior to test initiation to create the KCl test dilutions (0.64, 0.51, 0.41, 0.33, 0.26, 0.2, 0.16, 0 g/L ) and $CdCl_2$ test dilutions (0.25, 0.14, 0.08, 0.045, 0.025, 0.014, 0.008, 0 μM).The NAFC's were

prepared in a stock solution of 0.05 M NaOH. Solutions were prepared following Bartlett et al. (2017) methods, such that NAFC solutions (100 mg/L) and salt controls (0.05 M NaOH) were prepared 24 hours prior to test set up to allow for pH stabilization. NAFC treatments were made to a total concentration of 100 mg/L. The pH of these solutions was adjusted using 1 M HCl to 8.50 (± 0.1) 24 hours prior to test initiation. 2 hours prior to test initiation the pH of NAFC and salt control solutions was readjusted to 8.35 (± 0.1) and test treatments (100, 50, 20, 10, 5, 2 mg/L, salt control, and dechlorinated water control) were poured.

Plastic cups (500 mL) were used for inorganic KCl and $CdCl_2$ test solutions. Test replicates contained 5 cm x 5 cm square of cotton gauze, 400 mL of exposure solution, and 15 juvenile amphipods. Glass beakers (250 mL) were used for organic NAFC test solutions. Test replicates contained 2.5 cm x 2.5 cm square of cotton gauze, 200 mL of exposure solution, and 15 juvenile amphipods. *H. azteca* replicates were fed 1 mL of a 15 mg / 25 mL Tetra-Min slurry at test initiation and on day 4; survival was assessed at the end of the 7-day exposure. Two tests were completed for each test toxicant/mixture, with three replicates per control and treatment per test.

### 2.3.2 Low-Volume Method

Glass beakers (50 mL) were used for inorganic and organic NAFC exposures. Tests replicates contained 2.5 cm x 2.5 cm square of cotton gauze, 50 mL of treatment solution, and 10 juvenile amphipods. *H. azteca* were fed 1 mL of a 7.5 mg / 25 mL Tetra-Min slurry at test start and on day 4 to keep ammonia levels low. Survival was assessed at the end of the 7-day exposure. Two tests were completed for each test toxicant/mixture, with three replicates per control and treatment per test. Reduced volume test survival was compared to standardized test methods to determine if the results were similar, and if the adapted method could be used in place of the current standard protocol.

### 2.3.3 Statistical Analysis

Data were analyzed using R version 3.3.3 (R Core Team, 2017), RStudio version 1.0.136 (RStudio Team, 2016), and software package *drc* (Ritz et al., 2016). Data were fit to a four-parameter log-logistic model. The low-volume test and standardized test LC50 values from the same treatments were tested for relative potencies. If a ratio of the LC50 values, within a 95 % confidence interval, CI, was equal to 1 and p values above 0.05, than the two tests were considered similar (Ritz et al., 2006).

## 2.4 Results

The differences between the LC50s (Table 2-1) of the low-volume test and standard test were <1-fold for KCl, NAFC 2009, and NAFC 2011. Additionally, the LC50 values for each treatment's low-volume test and standardized test were not statistically different ($p = 0.34$, 0.73, and 0.48) for KCl, NAFC 2009, and NAFC 2011. The differences between the LC50s of the low-volume test and standard test were 2-fold for $CdCl_2$ and test methods were statistically different ($p = 0.03$). Additionally, the free metal ions in the $CdCl_2$ solution adhere to glass which remove them from solution. Therefore, the actual concentration of $CdCl_2$ is lower than the nominal concentration, and consequently the LC50 for $CdCl_2$ in the 50 mL glass beakers will appear to require a greater concentration in comparison to the plastic vessels. However, because the difference between the LC50s was 2-fold and the 95% CIs overlapped, it was determined that the differences in LC50s were not biologically meaningful between test methods. Mean survival of controls in all toxicity tests was 96 - 100 %, meeting the 90 % test validity criterion recommended for 96-h water-only tests (Environment Canada, 2013).

**Table 2-1:** LC50 of 7-day *H. azteca* reference toxicant and organic mixture tests for potassium chloride (g/L), cadmium chloride (μM), a naphthenic acid component fractionate from fresh tailings in the year 2009 and NAFC 2011 (mg/L).

| | **Treatment** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **$CdCl_2$ [μM]** | | **KCl [g/L]** | | **NAFC 2009 [mg/L]** | | **NAFC 2011 [mg/L]** | |
| **[mL]** | ***50*** | ***400*** | ***50*** | ***400*** | ***50*** | ***200*** | ***50*** | ***200*** |
| **LC50** | 0.124 | 0.101 | 0.293 | 0.301 | 30.40 | 31.47 | 36.21 | 38.43 |
| **Standard Error** | 0.010 | 0.006 | 0.006 | 0.005 | 2.42 | 1.82 | 2.38 | 2.13 |
| **Lower 95% Confidence Limit** | 0.105 | 0.088 | 0.281 | 0.290 | 25.67 | 27.91 | 31.53 | 34.25 |
| **Upper 95% Confidence Limit** | 0.143 | 0.114 | 0.305 | 0.311 | 35.14 | 35.03 | 40.88 | 42.61 |
| **% Difference** | 22.8 | | 2.66 | | 3.40 | | 5.78 | |
| **p value** | 0.03 | | 0.34 | | 0.73 | | 0.48 | |

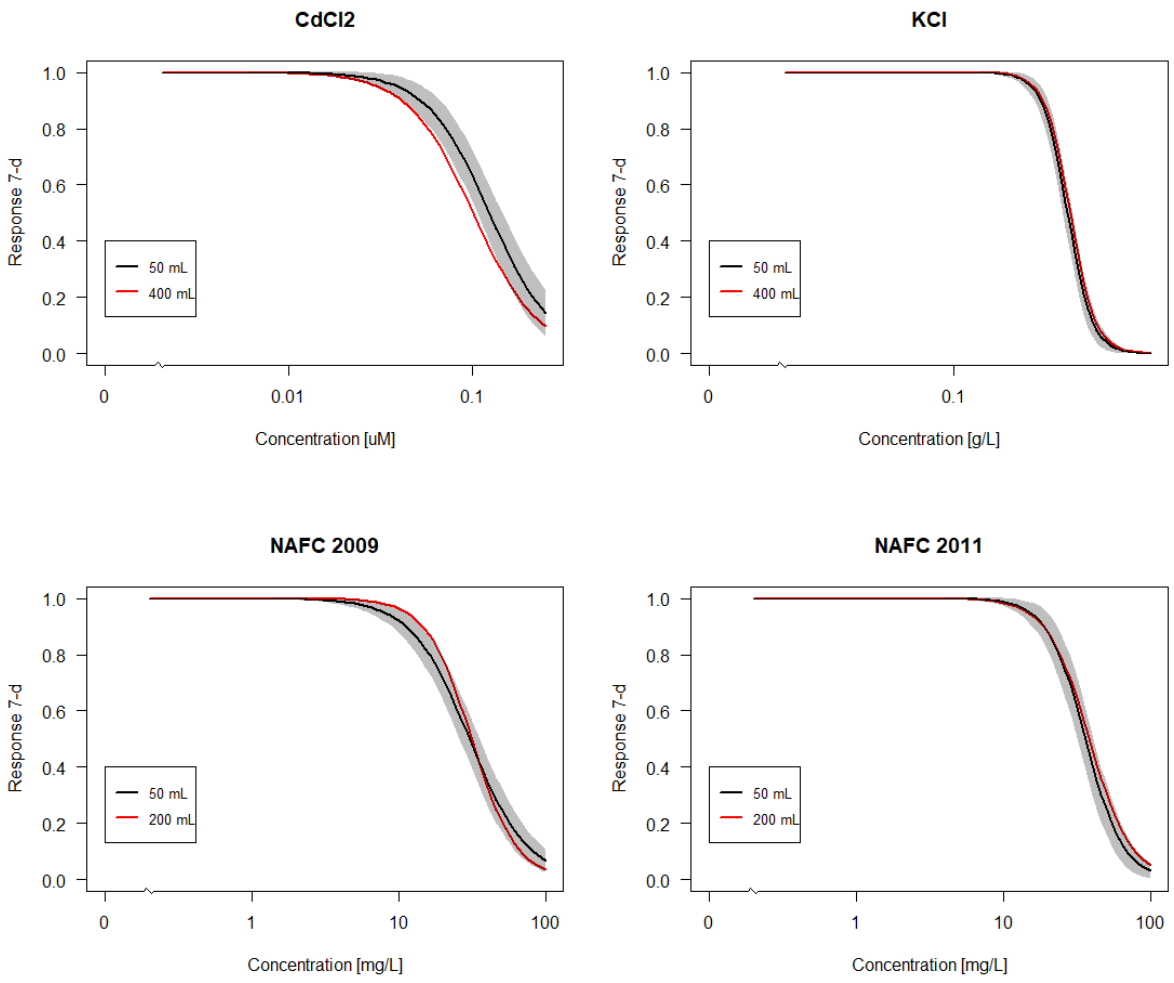


**Figure 2-1:** 7-d *Hyalella azteca* concentration response curve to inorganic toxicants cadmium chloride ($CdCl_2$), and potassium chloride (KCl), and organic mixtures of naphthenic acid fractionate from a fresh tailings source in 2009 (NAFC 2009) and 2011 (NAFC 2011). Grey shading is 95 % confidence interval region for the low-volume, 50 mL, test.

## 2.5 Discussion and Conclusion

The purpose of this study was to develop a new robust low-volume method for EDA applications which require biological testing with *H. azteca*. A reduced volume test method which utilized solution volumes of 50 mL with 10 juveniles was compared to standardized tests (Borgmann et al., 2005a; Environment Canada, 2013) using 400 mL and 15 juveniles. The

validity of the new low-volume method was analyzed by comparing the LC50s from the low-volume tests and standardized tests. The low-volume and standardized tests were completed in parallel for inorganic reference test solutions ($CdCl_2$ and KCl), and two organic mixture solutions (NAFC 2009, and NAFC 2011). The results from tests with standard inorganic reference toxicants ($CdCl_2$ and KCl) indicate that at reduced volumes of inorganic solutions, the *H. azteca* toxicity is comparable to the larger volume test methods and therefore could be used as an appropriate acute test. The LC50 for both NAFC organic mixtures, NAFC 2009 and NAFC 2011, were compared between the 50 mL test and the 200 mL test method used in past studies (Bartlett et al., 2017). The organic mixtures could not be aerated due to potential volatilization of organics, which was a concern as aeration is required as part of the test conditions to maintain sufficiently high dissolved oxygen and low ammonia concentrations for optimum amphipod survival. Concentration of ammonia was especially of concern in the 50 mL replicates due to minimal volume of solution per juvenile organism and no aeration to evaporate off excess ammonia. Water quality parameters were monitored at the start and end of the test to confirm that test mortality was due to organic materials in the NAFC solution and unrelated to water quality factors. The differences between the LC50s of the organic mixtures for the low-volume test and standard test were <1-fold NAFC 2009 ($p = 0.73$), and NAFC 2011 ($p = 0.48$).

The comparison of standard test methods to the adapted test method supports the acceptability of the reduced volume test as a suitable alternative to standard protocols when test solution volumes are limited. Additional replicates may be required due to the decreased number of organisms per replicate from the standard 15 - 20 juveniles to a total of 10 juveniles depending on research requirements. However, total volumes of additional replicates will be minimal in comparison to total volumes of standard test protocols. Overall, the development of a

reduced volume acute method for the *Hyalella azteca* bioassay is beneficial for EDA research with limited materials available interested in analyzing an environmentally relevant species.

# Chapter 3 : Effects-Directed Analysis of Bioactive Polar Organic Fractions from Industrial and Natural Sources

### 3.1 Summary

The characterization of the soluble polar organic compounds driving toxicity from OSPW and other bitumen-influenced waters is unknown because of the complexity of the inherent mixtures. Effects-directed analysis (EDA) is a tool used to identify the toxic compound(s) in toxic mixtures through means of mixture fractionation, and biological and chemical analyses. This study builds upon previous work by applying a new tier of EDA to the previously determined toxic polar organic fraction, 1°F3, that had been isolated from an aged tailings pond (Aged OSPW – 1) and three groundwater sites (Natural GW – 1, and Natural GW – 2, and OSPW influenced GW – 1). The polar organic fraction was sub-fractionated using reverse-phased high-performance liquid chromatography (HPLC) and separated in four secondary fractions (2°F) by polar characteristics; a recombined treatment (2°FR) was also created. Qualitative analysis was completed using a liquid chromatography time of flight mass spectrometer (LC-QToF/MS) in both ESI negative and positive ion modes. *Vibrio fischeri* (Microtox® assay) and *Hyalella azteca*, were exposed to the primary fraction, the isolated secondary fractions, and a recombined treatment. In the Microtox® assay 2°F3 and 2°F4 had the greatest inhibition within groundwater sites. Similarly, for the *H. azteca* assay *2*°F4 was the only secondary fraction for the *H. azteca* with reduced survival. The toxicity from the primary fraction was different from the secondary fractions but not different from the 2°FR for both OSPW Influenced GW – 1, and Natural GW – 2. These observations could possibly be due to the mixture of polar organics being more toxic as a whole, or, perhaps the class of compounds responsible for toxicity was split between two adjacent secondary fractions and therefore the toxic threshold was not reached. Through chemical analysis it was determined that each secondary fraction chromatogram was too complex to identify individual compounds, or to prioritize any compounds of interest. Future analyses will require a recombination of the

secondary fractions to determine if toxicity can be isolated in a single fraction and if multiple compounds are involved.

### 3.2 Introduction

The Canadian oil sands deposit of northern Alberta has an estimated 174 billion barrels of recoverable reserves of bitumen and crude oil (Alberta Energy and Utilities Board, 2016-2017; Allen, 2008). Bitumen is a mixture of viscous hydrocarbons which when broken down becomes a sought-after resource by many countries. The extraction and production of bitumen has rapidly expanded in the past few decades, from 1.20 million barrels of bitumen recovered per day in 2006 to 2.83 million barrels in 2017 (Alberta Energy and Utilities Board, 2007, 2017 - 2018). However, the extraction processes require a significant volume of water, 2 - 4.5 barrels of water per 1 barrel of crude oil collected (Alberta Energy and Utilities Board, 2016-2017; National Energy Board, 2015), which is collected from the Athabasca River watershed and is recycled. The recycled water is reused until it is highly saturated in salts, dissolved organics, metals, etc. These end of life oil sands process-affected waters (OSPW) are pumped into the large containments known as tailings ponds, where they will settle, to avoid direct interaction with the natural environment (FTFC, 1995b). Currently, the Government of Alberta has an established zero discharge policy in which there are no permits issued for the discharge of these wastes (Allen, 2008; Government Of Alberta, 2017). However, there have been recent reports of seepage of contaminants from tailings ponds into adjacent groundwaters and surface waters (Ferguson et al., 2009; Frank et al., 2014; Hewitt et al., 2019; Milestone et al., 2019; Ross et al., 2012), and there is concern that this seepage could have a negative impact on the local biota due to the toxicity associated with OSPW and the dissolved organic compounds therein (Anderson et al., 2012; Bartlett et al., 2017; Frank et al., 2009; Goff et al., 2013; Hagen et al., 2014; He et al., 2012; Kavanagh et al., 2013; Marentette et al., 2015a).

Tailings seepage and the potential for release of OSPW into the natural environment is of concern due to the high concentration of compounds in the settled OSPW (Allen, 2008; MacKinnon & Sethi, 1993). However, there exists a natural background of bitumen-influenced compounds in the waters of the Athabasca River watershed because waters flowing through the natural bitumen formations accumulate the soluble compounds. Current requirements for reclamation are to determine toxic drivers from tailings OSPW and natural bitumen-influenced waters, that could potentially have long term adverse effects on the natural biota and environment. Knowledge of the compounds causing toxicity can facilitate development of water quality guidelines with effective monitoring applications (Government of Alberta, 2018). Nevertheless, this task proves difficult due to the chemically complex mixtures of bitumen compounds because there are both natural and industrial processes within the region affecting soluble organic concentrations in the waters (Allen, 2008; Clemente & Fedorak, 2005; Hewitt et al., 2019). Therefore, it is challenging to discriminate between sources which ultimately affects understanding of what compounds are driving toxicity in the region.

Effects-directed analysis (EDA) is a tool used to identify the drivers of toxicity within complex mixtures of concern through fractionation techniques, toxicity assessments, and chemical analysis (Brack et al., 2016). Once a sample has been identified as being toxic, EDA utilizes a series of steps to identify the principal toxic components within complex mixtures using iterative steps of chemical fractionation guided by bioassay results (Altenburger et al., 2019; Brack et al., 2016). Due to the complexity of the dissolved organics in bitumen-influenced waters, EDA is likely to require multiple tiers to determine the compounds driving toxicity within these waters. The volume of materials available for fractionation is important for EDA as the amount of available material for analysis will decrease with each tier of fractionation. Recently, the dissolved organics from aged OSPW were extracted by means of a bulk SPE

fractionation method (Bauer et al., 2019), which generates three fractions based on polarity and pH. Fractions produced from aged OSPW and groundwaters influenced by both natural bitumen and OSPW contamination were evaluated using multiple species and showed the least polar organic fraction, 1°F1, and the most polar organic fraction, 1°F3, were the most acutely toxic (Bauer, 2018; Bauer et al., 2019; Frank et al., 2019b).

The objective of this study was to apply the next tier of EDA to multiple bitumen water sources previously investigated (Bauer et al., 2019; Frank et al., 2019a). Based on an effects-directed assessment, 1°F1 and 1°F3 required additional tiers of EDA for toxicity and chemical analysis. This current study focuses on the primary fraction 1°F3, a fraction in which the associated toxicity in some species was potentially due to higher abundance of oxygenated groups and degree of aromaticity (Bauer et al., 2019). The bioactive primary fraction, 1°F3, was chosen for further EDA analyses due to the mixtures' toxicity and chemical properties which make it a more optimal candidate for HPLC fractionation.

## 3.3 Materials and Methods

### 3.3.1 Bitumen-influenced Waters

Bitumen-influenced waters were collected from sites across the northern Alberta oil sands region. Two sites sampled, Natural GW – 1 and Natural GW – 2, are groundwaters which are far field, or, greater than 1 km from known OSPW sources and therefore not considered to have any OSPW influence. These far field sites only have naturally occurring bitumen-influenced compound (Bauer, 2018; Frank et al., 2014; Roy et al., 2016). Another site sampled, OSPW-influenced GW – 1, is groundwater that is near field, located within 200 m of a known OSPW source, and therefore contains some influence of OSPW (Bauer, 2018; Frank et al., 2014; Hewitt et al., 2019; Milestone et al., 2019; Roy et al., 2016). The final site, Aged OSPW – 1, is aged tailings pond water collected from a tailing's containment constructed in 1993 called Test

Pond 9. This site has not received fresh OSPW since its creation and is has aged over 20 years (Siwik et al., 2000). 2000L of Aged OSPW – 1 was collected in 2011, and 180 L of waters from these groundwater sites were collected in 2013. These waters were extracted using bulk SPE to collect three dissolved organic fractions with varied polarities. The fraction extracts were stored in a 5℃ fridge before injection into HPLC for further fractionation.

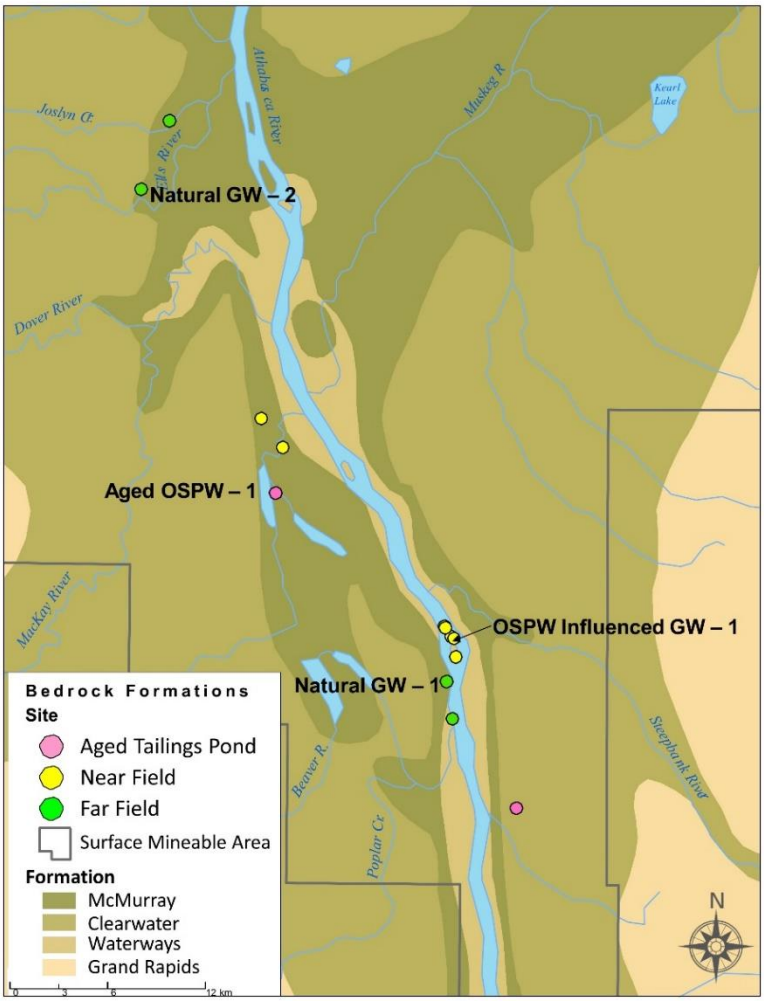


**Figure 3-1:** Map depicting sampling locations of the bitumen-influenced water sites, aged OSPW, naturally bitumen-influenced groundwaters and OSPW-influenced groundwaters and their proximity to anthropogenic OSPW sources in the Alberta oil sands region.

### 3.3.2 HPLC Method

Chromatography was performed on an Agilent 1200 series HPLC system (Agilent Technologies, Palo Alto, CA, USA). This system consists of a quaternary pump, micro vacuum degasser, autosampler, and a thermostatted column compartment. The method was developed on an analytical scale Agilent ZORBAX Eclipse XDB-C18 (4.6 x 150 mm, 5 μm) reverse-phase analytical column. The method was optimized to semi-preparative scale on an Agilent ZORBAX Eclipse XDB-C18 (9.4 x 150 mm, 5 μm) reverse-phase semi-preparative column using the equations $F_{semi} = F_{An} \frac{D_{Semi}^2}{D_{An}^2} \frac{dp_{an}}{dp_{semi}}$ and $t_{semi} = t_{an} \frac{L_{semi}}{L_{an}} \frac{D_{Semi}^2}{D_{An}^2} \frac{F_{an}}{F_{semi}}$ where F = flow rate, D = diameter, dp = particle size, t = gradient duration, semi = semi preparative scale, and an = analytical scale. The new duration times for the semi-preparative gradient were rounded to the nearest value. Polar organic fraction materials are detected using an ultraviolet diode array detector (UV DAD). The method developed separates the polar acid extractable organics of 1°F3 with a gradient mobile phase of water and methanol. Originally seven fractions were collected based on time, however, after preliminary toxicological and chemical analysis the decision to combine 2°F4 - 2°F7 was made due to no acute toxicity response to preliminary tests and no visual peaks on the LC-QToF in 2°F5, 2°F6, and 2°F7. In total four fractions were collected and analyzed (2°F1, 2°F2, 2°F3, and 2°F4), along with a treatment which recombined the secondary fractions (2°FR).

**Table 3-1:** Analytical scale high performance liquid chromatography method gradient profile for separation based on polarity characteristics.

| Time [min] | Flow Rate [mL/min] | % $H_2O$ | % MeOH |
|---|---|---|---|
| 0 | 1.0 | 95 | 5 |
| 2 | 1.0 | 95 | 5 |
| 20 | 1.0 | 0 | 100 |
| 30 | 1.0 | 0 | 100 |
| 31 | 1.0 | 95 | 5 |
| 36 | 1.0 | 95 | 5 |

**Table 3-2:** Semi-preparative scale high performance liquid chromatography method gradient profile for separation based on polarity characteristics.

| Time [min] | Flow Rate [mL/min] | % $H_2O$ | % MeOH |
|---|---|---|---|
| 0 | 4.2 | 95 | 5 |
| 2 | 4.2 | 95 | 5 |
| 32 | 4.2 | 0 | 100 |
| 48 | 4.2 | 0 | 100 |
| 50 | 4.2 | 95 | 5 |
| 58 | 4.2 | 95 | 5 |

The analytical HPLC method was scaled to a semi-preparative model to accommodate increased injection volumes, 100 µL to 500 µL, and output of fraction materials.

### 3.3.3 Biological Testing Sample Preparation

#### 3.3.3.1 Sample Preparation

HPLC fractions were collected and pooled to final test volumes based on whole water equivalent calculations for each primary fraction and site (Bauer, 2018). Solutions for Microtox® and *Hyalella azteca* assays were collected separately due to 3-fold differences in final solution preparations. Secondary fractions were dried with a rotary evaporation unit (BUCHI R-114/R-124) with the vacuum (BUCHI V-700/V-850) set to 340 bar and reduced to 73 bar in a 60°C water bath. After roto-evaporation, minimal amounts of water remained in each round bottom flask. Additional methanol (5 mL) was added to each flask to transfer solutions to a pre-weighed

vial for an additional 24 hours of drying using $N_2$ gas. Weights of dried fractions were recorded to consistent mass, such that weights were collected every 30 minutes until no change occurred, to confirm concentrations of each dried secondary fraction. The remaining residue was reconstituted in a 0.1 % methanol dechlorinated water solution based on the primary fractions whole water equivalents (WWE) (Table 1-1). For example, 17 mL of the Aged OSPW – 1 primary fraction extract is equal to 1 L of whole water so 0.85 mL of the primary fraction is equivalent to 50 mL of whole water.

A solution of 0.01 M NaOH, made by dissolving 400 mg of NaOH pellets in 1 L of dechlorinated water, was used to bring the pH of reconstituted secondary fractions to 8.35 ± 0.5 for the *H. azteca* tests. Primary fractions were prepared based on the method by Bauer et al. (2019). Bioassays were exposed to dechlorinated water control, a solvent control (0.1% methanol in dechlorinated water), 2°F1, 2°F2, 2°F3, 2°4, 2°F Recombined, and 1°F3 for each respective site.

#### 3.3.3.2 *Hyalella azteca*

A low-volume acute (7-d) test method adapted from a standard test method (Borgmann et al., 2005a; Environment Canada, 2013) was utilized for testing (Chapter 2). Initial water quality parameters (chloride, ammonia, dissolved oxygen (DO), pH, and conductivity) were recorded before solutions were transferred to respective testing beakers. Test replicates contained 2.5 cm x 2.5 cm square of cotton gauze, 50 mL of exposure solution (2°F1, 2°F2, 2°F3, 2°4, 2°F Recombined, and 1°F3), and 10 juvenile (2-10 day old) amphipods. *H. azteca* were fed 1 mL of a 7.5 mg / 25 mL Tetra-Min slurry at test initiation and on day 4, to keep ammonia levels low. Final water quality parameters per test replicate were recorded at test conclusion.

#### 3.3.3.3 *Vibrio fischeri*

Exposure solutions (solvent control, 2°F1, 2°F2, 2°F3, 2°4, 2°F Recombined, and 1°F3) were made to 3-fold WWE in a 0.1 % MeOH solution for testing. Microtox® analysis was performed on a Microtox® Model 500 Analyzer following Azur Environmental (1995) basic test guidelines. The impact to bioluminescence was identified in a 15-minute exposure.

#### 3.3.3.4 Statistical Analysis

Statistical data were analyzed using R version 3.3.3 (R Core Team, 2017) and RStudio version 1.0.136 (RStudio Team, 2016), using similar analyses to those used when assessing the primary fractions (Bauer et al., 2019). *H. azteca* and *V. fischeri* data were initially analyzed by comparing endpoints of treatment groups (1°F3, 2°F1, 2°F2, 2°F3, 2°F4, and 2°FR (where applicable)) to the control groups (solvent control for *V. fischeri* tests, and a pooled control for *H. azteca* tests) using one-way analysis of variance (ANOVA). The bioassay method for *V. fischeri* utilizes control water as a reference level (zero) to which data are normalized. Therefore, the solvent control treatment was compared as a control group. As for the *H. azteca* control groups, there was some evidence of difference in mean endpoint across the control and solvent control groups ($p = 0.07$). However, the control groups met survival conditions (> 90% survival) and therefore, control data were pooled and treated as a single group for comparison with fraction treatments.

ANOVA model assumptions were assessed via residual plots, Shaprio-Wilk's Test, and Levene's Test. All treatment groups (pooled control (*H. azteca*), solvent control (*V. fischeri*), 1°F2, 2°F1, 2°F2, 2°F3, 2°F4, and 2°FR) were compared within sites. Pairwise comparisons were assessed via Tukey's method when evidence of significant differences ($p \leq 0.05$) among treatment means were identified (Bauer, 2018). *H. azteca* data which violated model assumptions were assessed using the non-parametric Kruskal-Wallis Test. Pairwise comparisons were

completed using Dunn's Tests for treatment groups, with a Bonferroni adjustment for multiple comparisons (pooled control, 1°F2, 2°F1, 2°F2, 2°F3, 2°F4, and 2°F recombined).

Some analysis using parametric methods had mild to moderate violations of Shapiro-Wilk's test for normality and Levene's test for constant variance. This was partially due to low test replicates such that one data point was responsible for the violation. However, in situations where one of the model assumptions was mildly violated the other model assumption tests had no violations. Upon observation of the data, these violations were considered very mild and therefore the ANOVA method was deemed acceptable for the data. Additionally, *H. azteca* data for Aged OSPW – 1 had no observed response in survival endpoint data between treatments (above 99.5 % survival for all groups), therefore, no statistical method was used for analysis.

### 3.3.4 LC-QToF/MS Qualitative Method:

Qualitative chemical analysis was completed on the LC-QToF/MS following previously utilized methods (Bauer et al., 2019). All samples were dissolved in methanol for consistency with past procedures and analyses utilized a water and methanol gradient mobile phase. All secondary fractions collected from the HPLC were fully dried and brought up at the same WWE equivalents as the primary fraction to allow for of comparison. The analysis was completed in full scan negative ion mode (mass range 100-980) and in full scan positive ion mode (mass range 100 - 1100) using an LC-QToF 6520 (Agilent Technologies, Santa Clara, California, USA) under the following conditions: Gas temp 350°C, drying gas 10 L/min, nebulizer 35 psi, VCap 3000 V, Fragmentor 130 V, Skimmer 65 V, reference mass recalibration enabled (Bauer et al., 2019). The LC conditions were as follows: Column Poroshell 120 EC-C18, 3.0 x 50 mm 2.7 µm, Solvent A Water (0.1 % formic acid), Solvent B Methanol (0.1% formic acid), initial conditions 95% A for 2 minutes, to 100 % B at 20 minutes, hold until 30 minutes. Samples were injected

with 1 µL of labelled internal standard (9-anthracene-$d_9$-carboxylic acid, 84.4 pg/µL and decanoic-$d_{19}$ acid, 390 pg/µL) (Bauer et al., 2019).

## 3.4 Results

### 3.4.1 Secondary Fraction Dry Weights

Each primary fraction was dried and weighed prior to injection on HPLC and the secondary fractions were collected and evaporated to dryness. In addition to the dry weight data being used to assess if mass balance had been achieved, these dry weights were used to prepare the concentrations of each secondary fraction and contributed to the understanding of materials division between fractions. There is an overall loss of materials as the sum of the secondary fraction dry weights does not equal to the initial primary fraction weight. Additionally, overall loss of materials was observed to be greater for the Microtox® fractions than for the *H. azteca* fractions.

**Table 3-3:** Dry weight comparison of the primary fractions and secondary fractions required for a 7-day *H. azteca* exposure for each bitumen-influenced water source.

| **1°F3** | **Aged OSPW – 1 [mg]** | **OSPW Influenced GW – 1 [mg]** | **Natural GW – 1 [mg]** | **Natural GW – 2 [mg]** |
|---|---|---|---|---|
| Primary Fraction | 13.0 | 7.7 | 21.0 | 9.7 |
| **2°F#** | | | | |
| 1.0 | 4.0 | 2.2 | 5.4 | 5.0 |
| 2.0 | 3.8 | 1.2 | 5.2 | 1.1 |
| 3.0 | 2.8 | 1.9 | 6.8 | 0.7 |
| 4.0 | 1.0 | 1.8 | 4.0 | 1.8 |
| **Total [mg]** | **11.6** | **7.1** | **21.4** | **8.6** |
| **% Loss** | **11%** | **8%** | **0%** | **11%** |

| **1°F3** | **OSPW Influenced GW – 1 [mg]** | **Natural GW – 2 [mg]** |
|---|---|---|
| Primary Fraction | 8.6 | 13.0 |
| **2°F#** | | |
| Recombined | 7.7 | 13.0 |
| **% difference** | **10 %** | **0 %** |

**Table 3-4:** Dry weight comparison of the primary fractions and secondary fractions required for a 15 m Microtox® assay exposure for each bitumen-influenced water source.

| 1°F3 | Aged OSPW – 1 [mg] | OSPW Influenced GW – 1 [mg] | Natural GW – 1 [mg] | Natural GW – 2 [mg] |
|---|---|---|---|---|
| Primary Fraction | 5.2 | 3.2 | 7.9 | 4.0 |
| **2°F#** | | | | |
| 1.0 | 1.4 | 0.5 | 1.5 | 1.5 |
| 2.0 | 0.6 | 0.4 | 1.3 | 0.0 |
| 3.0 | 0.4 | 0.3 | 1.8 | 0.0 |
| 4.0 | 0.0 | 0.5 | 0.8 | 0.3 |
| **Total [mg]** | **2.4** | **1.7** | **5.4** | **1.8** |
| **% Loss** | **54%** | **47%** | **32%** | **55%** |

| 1°F3 | OSPW Influenced GW – 1 [mg] | Natural GW – 2 [mg] |
|---|---|---|
| Primary Fraction | 4.7 | 5.3 |
| **2°F#** | | |
| Recombined | 3.30 | 5.00 |
| **% difference** | **30 %** | **6 %** |

### 3.4.2 Chromatography

Total ion chromatograms (TIC) compared summed intensities of the positive ions (Figure 3-2) and negative ions (Figure 3-3) of the secondary fractions and primary fraction for each bitumen-influenced water. The secondary fractions contained polar organic constituents due to the composition of primary fraction, however each of the secondary fractions were separated such that 2°F1 contained the most polar materials and 2°F4 the least. Secondary fraction chromatograms from both ion modes were much less complex in comparison to each primary fraction, nevertheless, they each remained complex mixtures.

It was apparent in both ESI negative (Figure 3-2) and ESI positive (Figure 3-3) chromatograms for all sources examined that 2°F1 and 2°F4 were chemically distinct fractions. Furthermore, in ESI negative each 2°F2 appeared chemically distinct from 2°F4. Additionally, in ESI positive the peak response for 2°F4 (the least polar of the polar organic acids) was enhanced in comparison to 1°F3. The peak response from the primary fractions appeared to be recovered in the 2°FR of OSPW Influenced GW – 1 (Figure 3-2b, Figure 3-3b), and Natural GW – 2 (Figure 3-2d, Figure 3-3d), therefore the compounds within 2°F4 were likely suppressed within the matrix of the whole mixture. Additionally, the 2°FR chromatograms of OSPW Influenced GW – 1 and Natural GW – 2 fit within the primary fraction chromatogram. However, there was elevated peak response except for Natural GW – 2 peaks at 17.9 – 20.1 minutes. Differences in profiles between sites can be attributed to the differing chemical compositions associated with source locations. Visually the TIC profiles appeared similar between sites, with the exclusion of Natural GW – 2 (Figure 3-3d), the TICs were still too chemically complex to pinpoint compound similarities and composition.

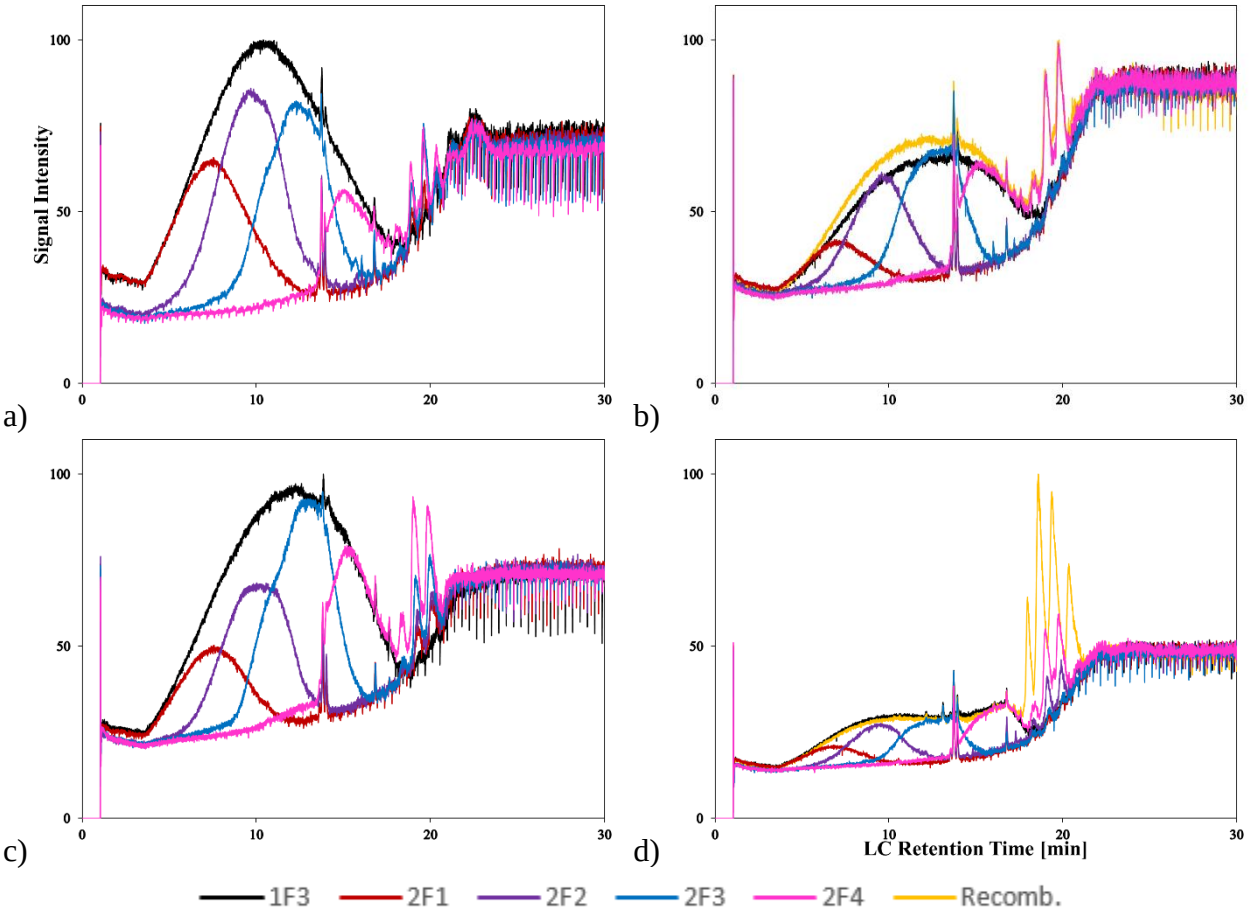


**Figure 3-2:** LC-QToF ESI negative total ion chromatograms (TICs) of signal intensity vs retention time for each site: a) Aged OSPW – 1, b) OSPW Influenced GW – 1, c) Natural GW – 1, and d) Natural GW – 2.

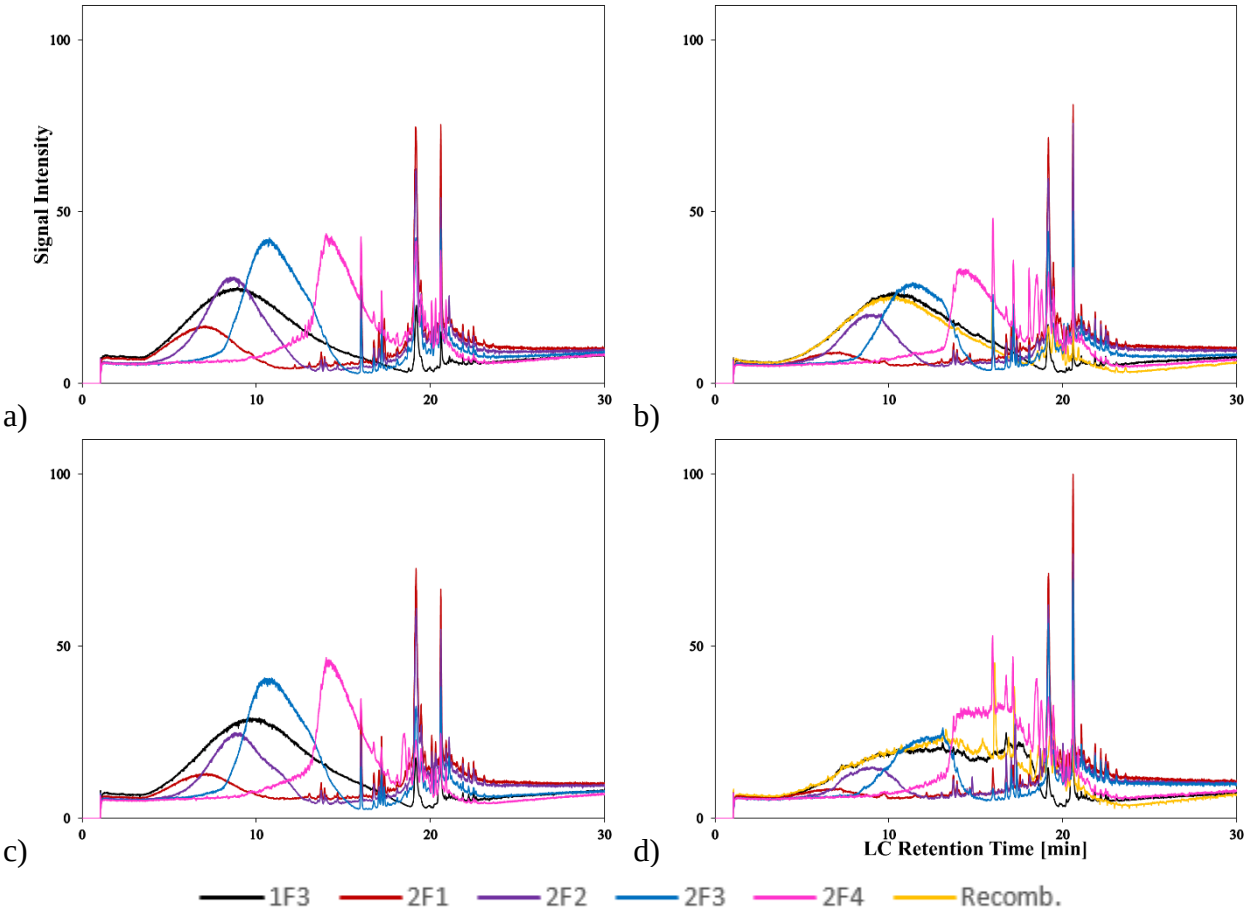


**Figure 3-3:** LC-QToF ESI positive total ion chromatograms (TICs) of signal intensity vs retention time for each site: a) Aged OSPW – 1, b) OSPW Influenced GW – 1, c) Natural GW – 1, and d) Natural GW – 2.

### 3.4.3 Microtox Assessments of Source and Secondary Fractions

*Vibrio fischeri* had no acute responses to any treatments at 1-fold whole water equivalents, however, acute responses were observed at 3-fold whole water equivalents (Figure 3-4). The consistent trend in the data with respect to bitumen-influenced groundwater source and aged OSPW was that the toxicity associated with each secondary fraction was less toxic than primary fraction and 2°FR exposures. In Aged OSPW – 1, 1°F3 was different from the other

treatments (48 % viability, $p < 0.001$). Toxicity was not recovered in secondary fraction treatments, which demonstrated no evidence of a difference from each other (77 – 87% viability, $p > 0.10$). There was minor evidence of a difference between 1°F3 and 2°FR ($p = 0.07$) which were the most toxic treatments at site OSPW influenced – 1 (29%, 40%). The was no difference in toxicity between 2°F3 (68% viability) and 2°F4 (70%, $p = 1.00$), but these secondary fractions were different from 2°F1 (87%, $p < 0.001$, $p = 0.002$) and 2°F2 (87%, $p < 0.001$, $p = 0.003$) which had no evidence of a difference ($p = 1.00$). Site Natural GW – 1 followed a similar trend in that 1°F3 had evidence of a difference from all other treatments ($p < 0.001$) and the most acutely toxic treatment (24% viability). There was no difference between the toxicity of 2°F3 (64% viability) and 2°F4 (62%, $p = 1.00$), but these fractions were different from 2°F1 (86%, $p < 0.001$, $p < 0.001$) and 2°F2 (82%, $p = 0.002$, $p = 0.001$) which did not have evidence of a difference ($p = 0.94$). At the Natural GW – 2 site there was moderate evidence of a difference in toxicity between 1°F3 (29% viability) and 2°FR (40%, $p = 0.054$) and evidence of a difference from each of the secondary fractions ($p < 0.0001$) and the solvent control ($p < 0.001$). 2°F2 (96% viability) was least toxic and different from 2°F1 ($p <0.001$), 2°F3 ($p < 0.001$), and 2°F4 ($p < 0.001$). There was no difference in the acute toxicity of 2°F1 (79% viability), 2°F3 (76%, $p = 0.99$), and 2°F4 (70 %, $p = 0.29$, $p = 0.70$). Pairwise comparisons of toxic responses were not compared between sites due to differences in chemistry between OSPW and natural bitumen-influenced compounds. Such as, the inclusion of mono-aromatic acids and sweeteners, etc, in industrial influenced source waters (Frank et al., 2014; Hewitt et al., 2019).

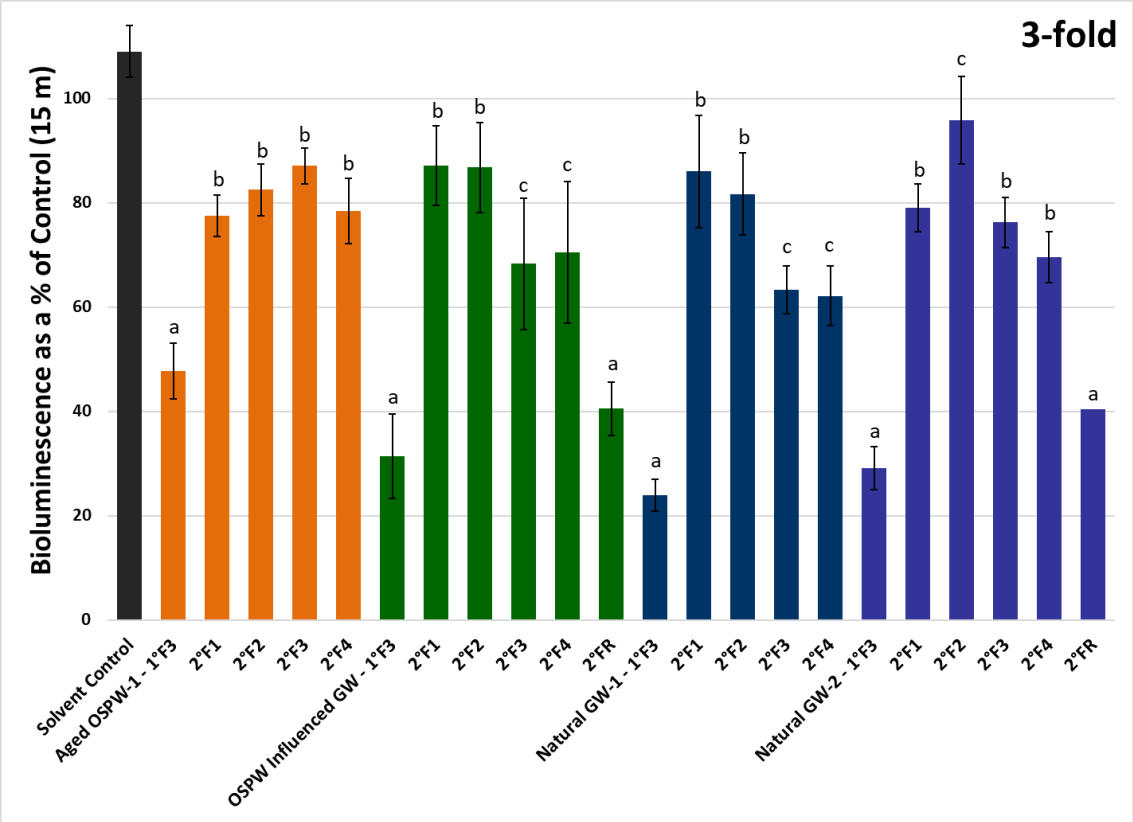


**Figure 3-4:** Percent inhibition of *Vibrio fischeri* (mean ± standard deviation) to secondary fractions for each bitumen-influenced water site: Aged OSPW – 1, OSPW Influenced GW – 1, Natural GW – 1, and Natural GW – 2. Pairwise comparisons are as indicated by the lowercase characters, were made within sites but not between sites. Exposure to primary and secondary fraction treatments was at 3-fold whole water equivalents of the original source water. Exposure fractions was from source water sites Aged OSPW – 1 (yellow), OSPW influenced GW – 1 (green), Natural GW – 1 (blue), Natural GW – 2 (purple).

### 3.4.4 *Hyalella azteca* Assessments of Source and Secondary Fractions

Similar to the Microtox® assay, there was less mortality from the individual secondary fractions than the primary fraction from each source type examined (Figure 3-6). Thus, a 2°FR treatment (2°F1 - 2°F4) was analyzed for the two sites with the greatest toxicity response from

the primary fraction, 1°F3: OSPW influenced GW – 1, and Natural GW – 2. There was no evidence of a difference in the toxicity of 1°F3 and 2°FR for Natural GW – 2 (0% survival, p = 1.00), and OSPW influenced GW – 1 (77.5%, p =1.00).

Aged OSPW – 1 had no observed response in survival endpoint data between treatments (above 99.5% survival for all groups), therefore, no statistical method was used for analysis. In OSPW Influenced GW – 1, *H. azteca* showed no response to fractions 2°F1, 2°F2, and 2°F3; survival in these fractions and controls was 99%. Survival in 2°F4 and 2°FR was 78% (p = 1.00), which was different than 2°F1, 2°F2, 2°F3 (each at 99%, p = 0.056, p = 0.0144). Additionally, 2°F4 and 2°FR had no evidence of a difference from 1°F3 (31%, p = 1.00). Natural GW – 1 had minor lethality in 1°F3 (93%) but had no evidence of a difference to 2°F4 (62% survival, p = 1.00). All treatments in Natural GW – 1 were had no evidence of a difference from each other (all p = 1.00), save for 2°F4 and the pooled control (p = 0.01). In Natural GW – 2, the *H. azteca* had no survival in both 1°F3 and 2°FR treatments (0% survival, p = 1.00). *H. azteca* showed no lethality to fractions 2°F1, 2°F2, and 2°F3; survival in these fractions and controls was 92 - 99%. Total lethality in 2°F4 (47% survival) was higher than 2°F1, 2°F2, 2°F3 (92 - 99%) and lower than 1°F3 (0%). However, there was weak evidence of a difference between 2°F4 and 2°F1 (p = 0.09), 2°F2 (p = 0.09), and no difference between 2°F4 and 2°F3 (p = 0.97), and 1°F3 (p = 1.00).

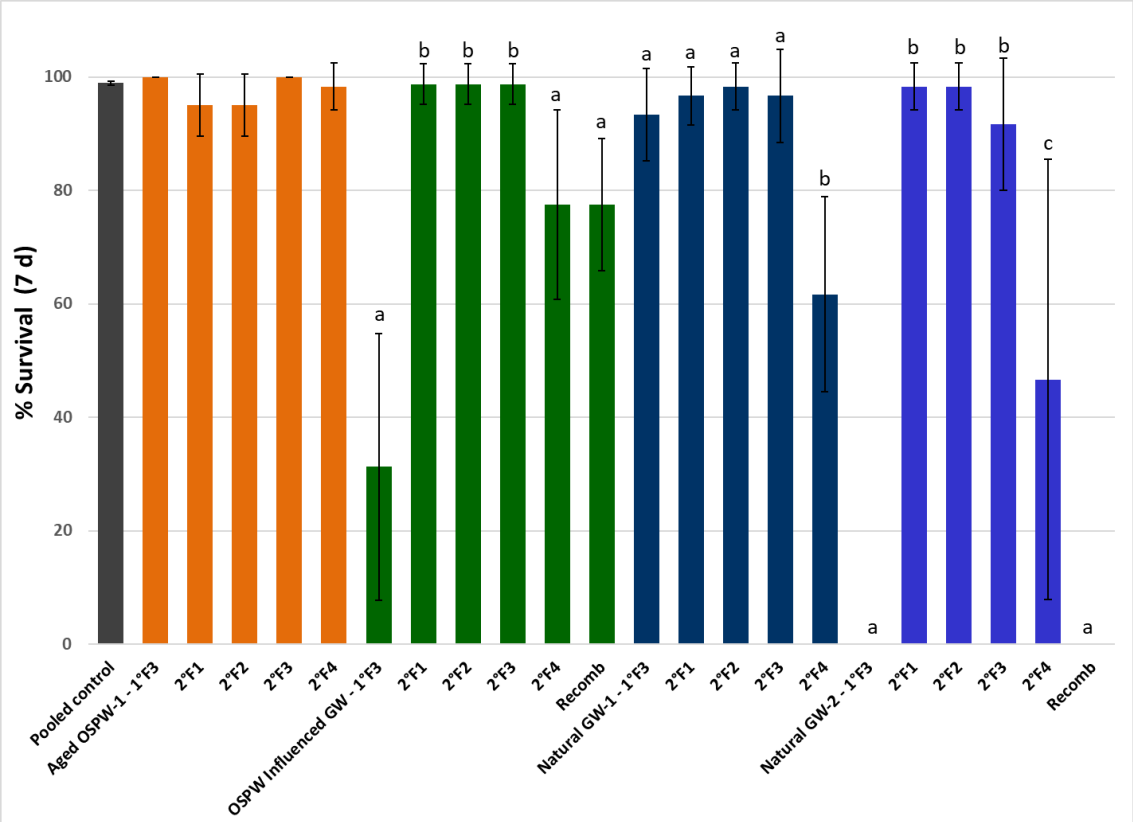


**Figure 3-5:** Percentage survival of *H. azteca* (mean ± standard deviation) exposed to control water, solvent control, 1°F3, 2°Fractions (2°F1 - 2°F4), and 2°FR treatments (recombination of 2°F1 - 2°F4). Controls were pooled (Pooled control) as there was no evidence of a difference between control groups (p = 0.07). Pairwise comparisons are as indicated by the lowercase characters, were made within sites but not between sites. No comparisons were made for Aged OSPW – 1 as each treatment met survivability conditions (>90% survival). Exposure fractions was from source water sites Aged OSPW – 1 (yellow), OSPW influenced GW – 1 (green), Natural GW – 1 (blue), Natural GW – 2 (purple).

### 3.5 Discussion

This study was an extension of previous EDA work with an aged tailings source and bitumen influenced groundwaters, both natural and OSPW influenced (Bauer, 2018; Bauer et al.,

2019). The primary objective of the study was to complete a second tier of EDA to continue the exploration of toxic drivers within the complex mixtures of polar soluble bitumen organics. The primary fraction from four sites that were previously analyzed, Natural GW – 1, Natural GW – 2, OSPW-influenced GW – 1, and Aged OSPW – 1, were to be sub-fractionated. The second objective, as per EDA, was to apply a non-targeted analysis to qualitatively complete an in-depth characterization of compounds in each secondary fraction for the four bitumen-influenced water sites. This chemical analysis compared secondary fraction profiles from toxic and non-toxic fractions. The objectives aim to continue the study of the toxic drivers from the bitumen influenced source waters that could potentially have long term adverse effects on the natural biota and environment. The knowledge of the compounds causing toxicity can help create water quality guidelines that have effective monitoring applications.

### 3.5.1 HPLC Method

Continuing with the second tier of EDA, the primary fraction of polar organics was fractionated using high-performance liquid chromatography (HPLC). HPLC was chosen because it can further decrease the complexity of a liquid sample due to separations based on subtle differences in the physical properties of the analytes, such as polarity. The compounds from the primary fraction introduced by the mobile phase were expected to interact with the stationary phase and elute off the column much more slowly than the mobile phase. For consistency with the fractionation method from the first tier of EDA, the polar organics within the primary fraction were sub-fractionated by polarity with a reverse-phase column. The duration of the gradient in conjunction with a mobile phase gradient of water and methanol was developed to achieve maximal chromatographic separations of each source water. However, due to the lack of chromatographic resolution of the unresolved complex mixture that comprised the primary fraction, there was no obvious location for the boundaries of each sub-fraction. Instead, 7 time-

based sub-fractions (2°F1 - 2°F7) were chosen such that each single sub-fraction would contain a comparatively less complex mixture of the totality contained within each primary fraction. After preliminary toxicological and chemical analysis, the decision to combine 2°F4 - 2°F7 was made due to lack of acute toxicity response from the *Vibrio fischeri,* and visual peaks on the LC-QToF in 2°F5, 2°F6, and 2°F7. In total, four secondary fractions were collected from the primary fraction: 2°F1 (the most polar of the organics), 2° F2, 2° F3, and 2° F4 (least polar of polar organics). It is likely that the largest polar organic species, $O_5$, $O_5S$, $O_{5+,}$ elute from column in the final fractions, however, chemical analysis is required to confirm overall speciation of these secondary fractions.

### 3.5.2 Qualitative Analysis

The primary and secondary fractions from each bitumen-influenced water source were analyzed using the LC-QToF/MS in both ESI positive ion mode and ESI negative ion mode. Multiple observations can be derived from analysis of the TIC from ESI positive and ESI negative within individual sites. Each secondary fraction was determined to be too complex to complete a targeted analysis, or to discern different compounds between fractions. Therefore, unknown compounds can not yet be determined, nor can a full in-depth characterization of these constituents be completed. Due to this, more fractionation tiers will need to be applied to further decrease the mixture complexity and therefore improve upon the chromatographic resolution. Secondly, it was apparent in both ESI positive and ESI negative chromatograms that 2°F1 and 2°F4 are chemically distinct fractions, because of the separation in chromatographic retention times between eluting components. Similarly, in ESI negative each 2°F2 appeared chemically distinct from 2°F4. These observations impart the importance of using multiple methods for analyses due to the detection of different analytes. Multiple ion sources serve as a complementary means to profile such complex mixtures. The response of 2°F4, the least polar of

the polar organic acids, in ESI positive is enhanced in comparison to the primary fraction. The response in 2°FR is similar to the primary fraction, suggesting that the compounds within 2°F4 are perhaps suppressed in the matrix within the whole mixture. This response is only apparent with the positively ionized compounds and not observed in ESI negative. Finally, Natural GW – 2 was the most difficult to analyze due to variability of pressure during the run, and drift of internal standard peak retention times, thought to be a result of the high abundance of dissolved organics and visual oil globules upon sampling (Bauer, 2018; Frank et al., 2014).

### 3.5.3 Toxicity Analysis

The biological tests selected were based on previous toxicity responses to the primary fraction's polar organics. To optimize the limited volume of extract created with each additional tier of EDA the bioassay selection was narrowed down to the most sensitive organism(s). In a previous study, *Hyalella azteca* was highly responsive to polar organics of the primary fraction and *Vibrio fischeri* had relatively comparable responses to this fraction at concentrations of 3-fold whole water equivalents (Bauer et al., 2019). EDA benefits from the use of *Vibrio fischeri* (Microtox® assay) because it requires minimal volumes and aids high throughput (Brack et al., 2016). However, *V. fischeri* is a marine bacterium that is not an environmentally relevant organism to the oil sands region and the Athabasca watershed and should be used only as a surrogate, or preliminary method for toxicity determination. *H. azteca* is native to the Athabasca watershed and very responsive to OSPW materials (Bartlett et al., 2017; Bauer et al., 2019), though the volume requirements for standardized tests (600 – 800 mL) are too great for many effects-directed applications. The bulk primary fractionation created large quantities of materials, and therefore, there was no issue meeting volume requirements of the standardized *H. azteca* biological test protocols (Bauer et al., 2019; Environment Canada, 2013). However, the creation of equal volumes of secondary fractions is time and cost prohibitive; therefore, the recently

developed low-volume *H. azteca* assay (Chapter 2) adapted from Environment Canada (2013) was used for this second tier of EDA.

Primary and secondary fraction dry weights (Table 3-4, Table 3-5) were recorded to determine the percent recovery of the secondary fractions by a mass balance. The loss of toxicity in the secondary fractions for *V. fischeri* and *H. azteca* tests could be attributed to the loss of mass. However, the loss of toxicity in the secondary fractions was not attributed to loss of materials from HPLC fractionation as the 2°FR treatments were not different from the primary fractions. Additionally, there was good recovery in the mass balance for *H. azteca* analyses (Table 3-3) and acceptable recovery for *V. fischeri* analyses (Table 3-4).

### 3.5.4 Microtox® assay

The bioluminescence response for each bitumen-influenced source water individual secondary fraction was less toxic compared to the primary fractions. However, due to sharing of compound classes in adjacent fractions (Figure 3-2, Figure 3-3), it is possible that toxic materials were below threshold for toxicity in the individual fractions. Additionally, the 2°FR treatment toxicity was much greater in comparison to the individual secondary fractions, suggesting the mixture of polar organics was more toxic as a whole in comparison to the individually separated components.

Furthermore, the trend of the data with respect to source of bitumen-influenced groundwaters sites and aged OSPW was that the toxicity associated with each primary fraction, was not recovered in any secondary fraction. With regard to the individual secondary fractions from each source water, there was no obvious toxicity trend that was comparable between bitumen-influenced waters. This may partially be due to the complexity of compounds resulting from different bitumen sources within the oil sands region. Bitumen-derived organics and AEOs understandably lack uniformity across the McMurray oil sands formation, therefore, it is

understood that the organic mixtures vary due to factors such as differences in source materials, location of groundwaters, age of waters, etc (Frank et al., 2016; Headley et al., 2011). However, apart from Aged OSPW – 1, 2°F3 and 2°F4 are consistently the individual secondary fractions with the lowest bioluminescence response.

### 3.5.5 *Hyalella azteca*

Similar to *Vibrio fischeri,* the toxicity response of *Hyalella azteca* to the primary fraction from each bitumen-influenced source water was not recovered in any of the individual secondary fractions. This suggests that the mixture is more toxic as a whole in comparison to the individually separated components. Overall, *H. azteca* had minimal toxic responses to the secondary fraction treatments such that 2°F4 was the only individual secondary fraction which invoked an acute response in sites OSPW influenced GW – 1, Natural GW – 1, and Natural GW – 2. Natural GW – 2 had the most pronounced response in comparison to the other GW sites and Aged OSPW – 1. ESI-HRMS analysis of the primary fraction from this site by Bauer (2018) indicated a high contribution of polyoxygenated ($O_{4+}$ species). However, further analysis of 2°F oxygen speciation is required to make any basic conclusions about the sensitivity of *H. azteca* to these polar organic compounds.

## 3.6 Conclusion

The objective of this current study was to complete a second tier of EDA on the toxic primary fraction containing polar organics from four sites: Natural GW – 1, Natural GW – 2, OSPW-influenced GW – 1, and Aged OSPW – 1. The primary fraction was sub-fractionated by reverse-phase HPLC to develop four secondary fractions, where 2°F1 is the most polar and 2°F4 is the least polar of the polar organic materials. Non-targeted chemical analysis on the primary and secondary fractions from bitumen-influenced waters were analyzed using LC-QToF/MS in both ESI negative and ESI positive ion modes. Observations of the TICs from both analyses

made it apparent that 2°F1 and 2°F4 are chemically distinct fractions. However, the secondary fraction profiles were determined to be too complex to complete a targeted analysis, or to discern different individual compounds between fractions.

Biological testing of the primary fraction, secondary fractions, and a recombined treatment was completed with the *Vibrio fischer* (Microtox® assay), and reduced volume *Hyalella azteca* assay. The acute response of the primary fraction was captured in the 2°FR treatments of OSPW Influenced GW – 1 ($p = 0.07$) and Natural GW – 2 ($p = 0.054$) in the Microtox® assay and 2°FR treatment of Natural GW – 2 ($p = 1.00$) in the *H. azteca* test. The acute responses of the individual fractions displayed no evidence of a difference to the primary fraction, however, 2°F3 and 2°F4 had the greatest response within groundwater sites. Similarly, for the *H. azteca* there was no toxicological response in 2°F1, 2°F2, 2°F3, but there was an acute response in 2°F4 for OSPW Influenced GW – 1 (78 % survival), Natural GW – 1 (62% survival), and Natural GW – 2 (47% survival). The recovery of toxicity in recombined exposures suggests that the toxicity of polar organic bitumen materials was due to mixture effects versus a single compound or class, which in turn suggests the possibility that multiple compounds are responsible for the effects and toxicity responses observed with *Vibrio fischeri* and *Hyalella azteca*. However, it could be possible that polar organic compounds driving toxicity were split between adjacent secondary fractions. It is also possible that the minor losses in the fractionation and preparation (Table 3-4, Table 3-5), contributed to these observations. Differences in abundances of materials, as observed in the dry weights (Table 3-4, Table 3-5) in the secondary fractions between the *H. azteca* and Microtox® materials may be due to complexity and heterogeneity of mixtures. Additionally, differences may be due to total amounts of material, such that the Microtox assay had less materials and was therefore more susceptible to minor losses from handling.

It is possible that the larger polar organic compounds are driving toxicity in the bitumen influenced waters due to the toxicity response in 2°F3 and 2°F4 for the *V. fischeri*, and 2°F4 for *H. azteca*. However, given that the toxicity of the primary fractions was greater than the secondary fractions the second tier of EDA will require re-evaluation to verify if the toxicity is split between adjacent secondary fractions. Additionally, further chemical analysis is required to determine the speciation and chemical characteristics of the compounds in the secondary fractions. These additional analyses will provide a deeper understanding of the polar organic compounds driving the toxicity within the soluble organic portion of bitumen influenced organics, which can be applied to future monitoring programs, and industrial reclamation strategies.

# Chapter 4 : Conclusions and Recommendations

### 4.1 Sub-fractionation and Qualitative Analysis

A second tier of EDA was applied to the polar organic fraction, 1°F3, from four bitumen-influenced water sources (aged OSPW – 1, OSPW Influenced GW – 1, Natural GW – 1, and Natural GW – 2), to advance the understanding of the compounds causing acute toxicity to freshwater aquatic biota (Bauer et al., 2019). The objective of this study was to further simplify the polar primary fraction by sub-fractionation to further target the polar organic compounds which are driving toxicity. A new HPLC method using a reverse-phase $C_{18}$ column and $H_2O$/MeOH gradient was created to separate the polar organic fraction based on polarity. Four secondary fractions were created where 2°F1 was the most polar and 2°F4 was the least polar of the polar organic compounds in the primary fraction. Solutions of each primary fraction were dried, weighed, and reconstituted prior to injection in order to complete a full mass balance. After fractionation, individual secondary fractions were brought to dryness using roto-evaporation and $N_2$ and weighed so the concentration of each fraction could be verified, and a full mass balance could be completed. Final mass measurements showed a minor loss of mass due to the fractionation and drying processes, that did not account for toxicity losses in biological assays. Prior to further analysis, dried fractions residue was reconstituted in 100 % methanol for chemical analysis or a 0.1 % methanol dechlorinated water solution based on the primary fractions whole water equivalents (Table 3-3).

Non-target qualitative analysis was completed using an LC-QToF in ESI negative and positive ion mode; ESI negative ion mode has been used for many oil sands organics' qualitative studies (Bauer et al., 2019; Brunswick et al., 2015; Headley et al., 2002). The chromatograms for the secondary fractions fit under the primary fraction chromatogram; there was no elevated peak response except for Natural GW – 2 peaks at 17.9 – 20.1 minutes. It was not known if these

peaks were created during the run cycle on the HPLC, or if they are natural to each fraction but have an increased peak signal in the 2°FR treatment.

ESI positive ionization mode was also chosen for analyses to provide additional insight into potential differences between the secondary fractions due to visualization of positively ionized compounds. The positive TIC for each site in Figure 3-3 shows a peak height response in 2°F4 that surpasses the original primary fraction, 1°F3. Additionally, 2°F3 from Aged OSPW – 1 and Natural GW – 1 TIC peak heights surpass 1°F3 as well. The response of these secondary fractions may be due to suppression from the matrix in the mixture, especially because the recombined treatments recover the 1°F3 response. This response does not appear in ESI negative and was therefore only apparent when the compounds are positively charged. Further evaluations of the ESI positive ionization mode TIC reveals the fractions 2°F1 and 2°F4 were chemically distinct. The use of two qualitative analyses was important because 2°F1 and 2°F2 were chemically distinct from 2°F4 in ESI negative ionization mode. Therefore, from comparisons of positive and negative ion TIC, it can be concluded that 2°F1 and 2°F4 were chemically distinct within groundwater sites.

In conclusion, the results of the TICs showed that the secondary fractions were less complex in comparison to their primary fraction; however, each secondary fraction was still too complex to determine unknowns or to complete an in-depth characterization of compounds. An additional tier of EDA must be performed to further fractionate these complex mixtures to determine the polar organic compounds responsible for toxicity to freshwater organisms.

## 4.2 Toxicity Analysis

### 4.2.1 Development of low-volume bioassay using *H. azteca*

The availability of materials for toxicity assessment and chemical analysis decreases with each additional tier of EDA. For this reason, the choice of bioassay can be limited by availability of testing solutions as the ideal biological test organism may have large volume requirements that are incompatible with EDA. The *Hyalella azteca* is a freshwater amphipod, common to many Canadian freshwaters and the Athabasca watershed, and has been demonstrated to be sensitive to dissolved organics from bitumen-influenced waters (Bartlett et al., 2017; Bauer et al., 2019). A reduced volume method was developed based on standard 7-day acute water-only *H. azteca* tests (Borgmann et al., 2005a; Environment Canada, 2013). The robustness and viability of this low-volume test method were verified by analysis of two inorganic reference toxicants, and two organic treatments. The effectiveness of this new low-volume acute method was demonstrated by a comparison of LC50's from standardized tests that were conducted concurrently.

Bioassays with the two inorganic reference toxicants, $CdCl_2$ and KCl, were completed following a dilution series of 70% and 80%. The two organic treatments, NAFC 2009 and NAFC 2011, were completed following preparations as detailed in Bartlett et al. (2017). The LC50's of both the low-volume test and standardized test for KCl, NAFC 2009, and NAFC 2011 were within a 1-fold difference of each other, 95% confidence limits overlapped, and not statistically different ($p > 0.05$). The differences between the LC50s for $CdCl_2$ test methods were statistically different ($p = 0.03$). However, the free metal ions in the $CdCl_2$ solution adhere to glass which remove them from solution. Therefore, the actual concentration of $CdCl_2$ is lower than the nominal concentration, and consequently the LC50 for $CdCl_2$ in the 50 mL glass beakers will appear to require a greater concentration in comparison to the plastic vessels. Due to the 2-fold

difference between the LC50s and the overlapping 95% CIs, there was strong evidence that the LC50s were not biologically different between test methods.

### 4.2.2 Toxicity analysis for EDA

This study continued toxicity assessments on polar organic materials from bitumen-influenced waters with the utilization of a second tier of EDA. Bioassays selected for analysis were *Hyalella azteca* and *Vibrio fischeri* (Microtox assay) due to their sensitivity to the polar organic fraction. *H. azteca* was highly sensitive to the polar organic materials and the Microtox response at 3-fold whole water equivalents had similar relative responses (Bauer et al., 2019). Bioassay fraction volumes were prepared according to the whole water equivalents of the primary fractions for each groundwater sites (Bauer, 2018). Solutions were prepared for Microtox analysis by reconstituting the dried fractions in 0.1 % methanol and dechlorinated water and equilibrated for minimum 2 hours prior to test initiation. Solutions were prepared for *H. azteca* analysis by reconstituting the dried fractions in 0.1 % methanol and dechlorinated water 24 hours prior to test initiation. The pH of solutions was adjusted using 1 M HCl to 8.50 (± 0.1) 24 hours prior to test initiation, and the pH was readjusted to 8.35 (± 0.1) 2 hours prior to test initiation.

The bioluminescence response in *Vibrio fischeri* was not significantly different between individual secondary fraction exposures. However, apart from Aged OSPW – 1, it could be suggested that 2°F3 and 2°F4 were consistently the secondary fractions with the lowest bioluminescence response. Yet there was no obvious toxicity trend with the *Vibrio fischeri* for the secondary fractions between bitumen-influenced waters. This may partially be due to the complexity of compounds within the Oil Sands and differences of organic constituents at each site. *H. azteca* had no apparent toxicological response in 2°F1, 2°F2, 2°F3, but there was an acute response in 2°F4 for OSPW Influenced GW – 1 (78% survival), Natural GW – 1 (62%

survival), and Natural GW – 2 (47% survival). Additionally, the site Natural GW – 2 was overall the most toxic site in comparison to the other GW sites and Aged OSPW – 1. ESI-HRMS analysis of the 1°F3 from this site by Bauer (2018) identified a significant contribution of $O_4$, and $O_{4+}$ species. *H. azteca* may be most sensitive to these species, however, further analysis of 2°F4 oxygen speciation is required to make any basic conclusions about the sensitivity of this organism.

The toxicity between the primary fraction and the individual secondary fraction treatments for each source water in both the *V. fischeri* and *H. azteca* were found to be different. However, there is some evidence that the acute response of the primary fraction was captured in the 2°FR treatments of OSPW Influenced GW – 1 ($p = 0.07$) and Natural GW – 2 ($p = 0.054$) in the Microtox® assay and OSPW influenced GW – 1 ($p = 1.00$) and 2°FR treatment of Natural GW – 2 ($p = 1.00$) in the *H. azteca* test. This suggests that the respective toxicities of the components in each secondary fraction were below threshold, or the overlap between fractions reduced the toxic concentrations in adjacent fractions below threshold (Figure 3-2, and Figure 3-3).

### 4.3 Recommendations

Due to the recovery of toxic effects in the 2°FR recombined treatments from OSPW influenced GW – 1 and Natural GW – 2, and lack of comparable toxicity in the individual secondary fractions, it is recommended to combine the fractions 2°F1 – 2°F2, 2°F2 – 2°F3, and 2°F3 – 2F°4 to determine how the toxicity is divided between fraction pairs. If toxicity is recovered in one of the combined fractions, a next tier of EDA will be required for the toxic fraction, especially as the secondary fractions are chemically complex. This next tier of EDA could apply supercritical fluid chromatography (SFC) for increased chromatographic separation of compounds. Additionally, further exploration of the classes of compounds associated with

toxicity, using ESI-HRMS, should be explored. However, it may be possible that toxicity is driven by the mixture of polar organic compounds and the new secondary fractions will not recover this toxicity.

Finally, due to the age of groundwaters and age of the tailings from Aged OSPW – 1, the compounds from these sites are not directly comparable to fresh bitumen compounds because of the decomposition of the organics (Frank et al., 2016; Grewer et al., 2010). It would be of interest to subject fresh OSPW materials to the bulk extraction method and subsequent sub-fractionation of primary fraction for comparison with aged OSPW and groundwaters (Bauer et al., 2019).

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*Appendices*

## Appendix A: Supporting Information for Chapter 3

**Table A-1:** Degrees of freedom and F values from 1-way ANOVA of Microtox® assay bioluminescence data for bitumen-influenced water sites: Aged OSPW – 1, OSPW Influenced GW – 1, Natural GW – 1, and Natural GW – 2.

| Source Water Site | Degrees of Freedom (N-1) | F Value | P Value |
|---|---|---|---|
| Aged OSPW – 1 | 5 | 62.44 | 6.01E-15 |
| OSPW Influenced GW – 1 | 6 | 98.01 | <2E-16 |
| Natural GW – 1 | 5 | 86.72 | <2E-16 |
| Natural GW – 2 | 6 | 160.6 | <2E-16 |

**Table A-2:** Degrees of freedom and F values from 1-way ANOVA of *Hyalella azteca* survival for bitumen-influenced water sites: OSPW Influenced GW – 1, Natural GW – 1, and Natural GW – 2. Aged OSPW – 1 was not analyzed due to each treatment passing survival conditions (>90% survival).

| Source Water Site | Degrees of Freedom (N-1) | F Value | P Value |
|---|---|---|---|
| OSPW Influenced GW - 1 | 6 | 52.8 | <2E-16 |
| Natural GW - 1 | 5 | 4.076 | 0.00492 |
| Natural GW - 2 | 6 | 72.75 | <2E-16 |

**Table A-3**: Degrees of freedom and P values from Kruskal-Wallis test of *Hyalella azteca* survival data for bitumen-influenced water sites: OSPW Influenced GW – 1, Natural GW – 1, and Natural GW – 2.

| Source Water Site | Degrees of Freedom (N-1) | P Value |
|---|---|---|
| OSPW Influenced GW - 1 | 6 | 9.13E-11 |
| Natural GW - 1 | 5 | 0.02066 |
| Natural GW - 2 | 6 | 2.44E-07 |

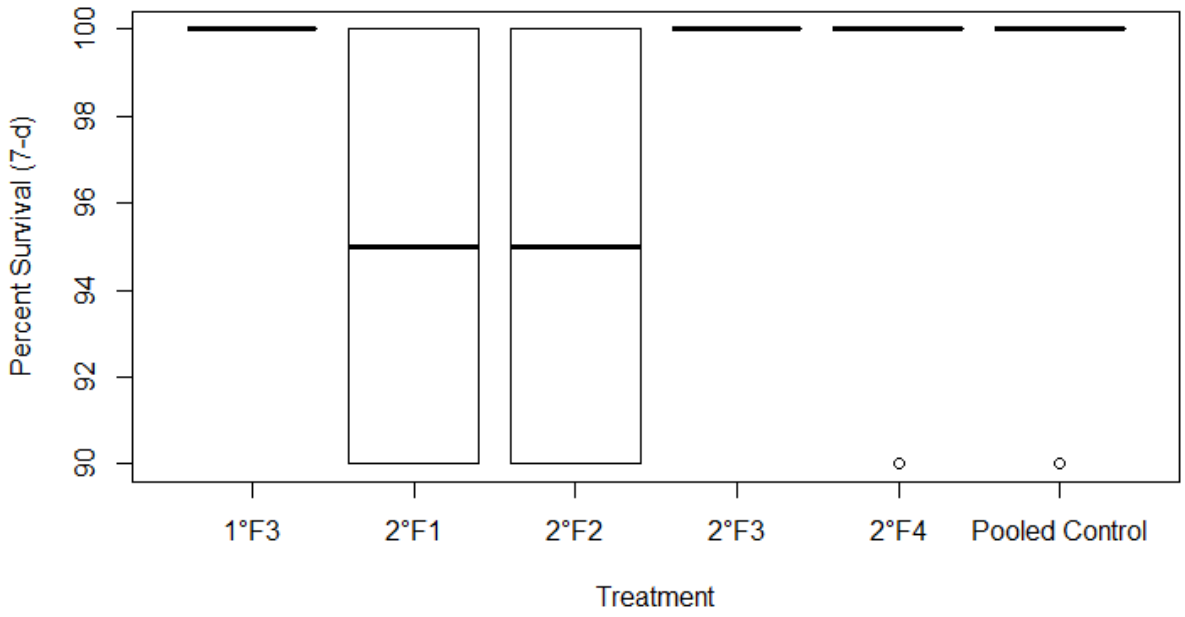


**Figure A-1:** Boxplot of percent survival of *H. azteca* to Aged OSPW – 1 primary fraction, secondary fractions, and pooled control. Each treatment meets survival conditions of the control (>90% survival), and therefore lacks variability.

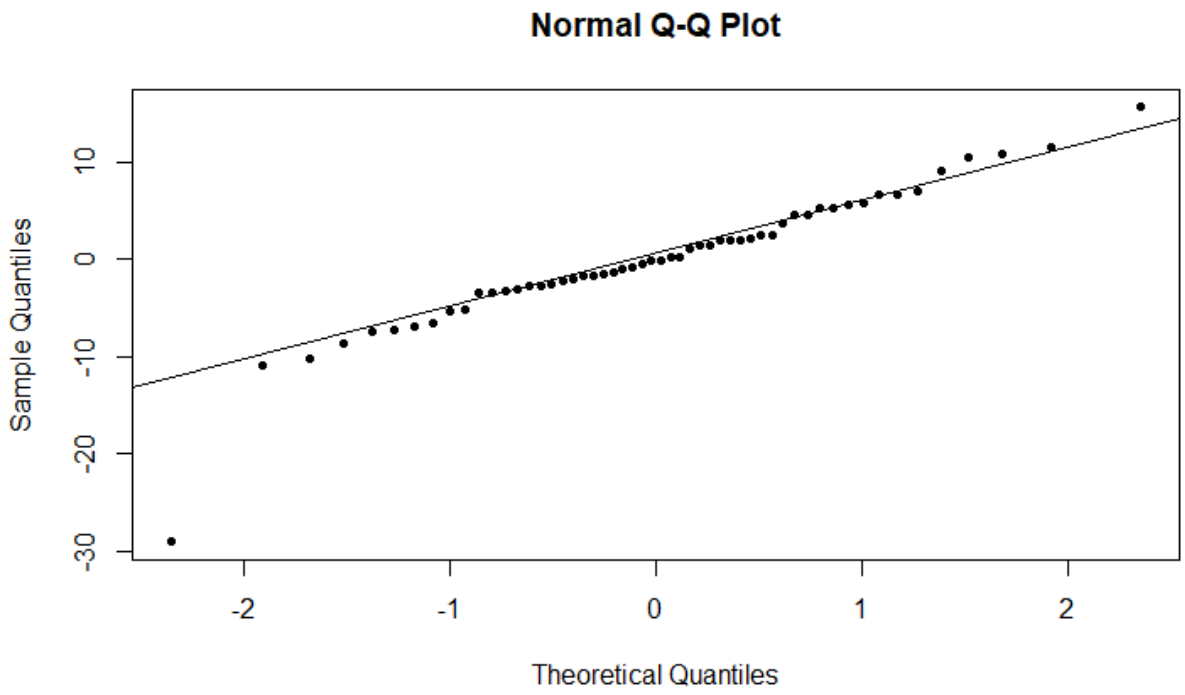


**Figure A-2:** Fitted residual plot of Natural GW - 2 Microtox assay data. The one outlier causes the rejection of the null hypothesis for the Shapiro-Wilks test (p = .001) and therefore the data does not have a normal distribution. Besides this point, the data is normally distributed along the line of best fit.

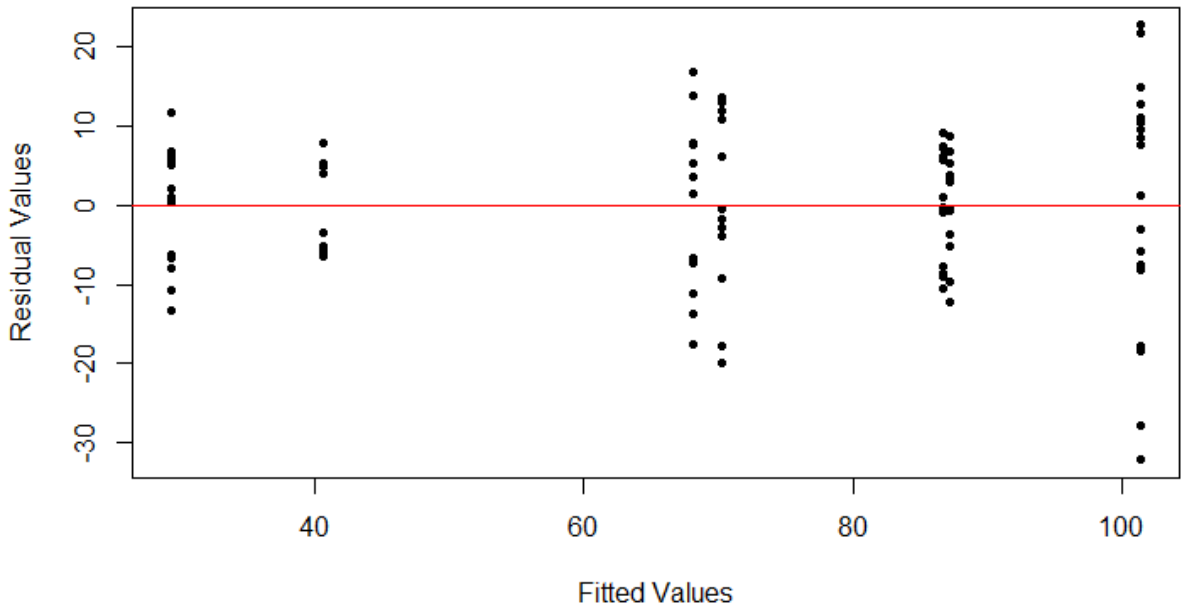


**Figure A-3*:*** Residual versus variance plot for OSPW Influence GW – 1 Microtox assay data. Due to the variance of solvent control data above 100, the null hypothesis of Levene's test is rejected ($p = .002$) and the data does not uphold consistent variance.

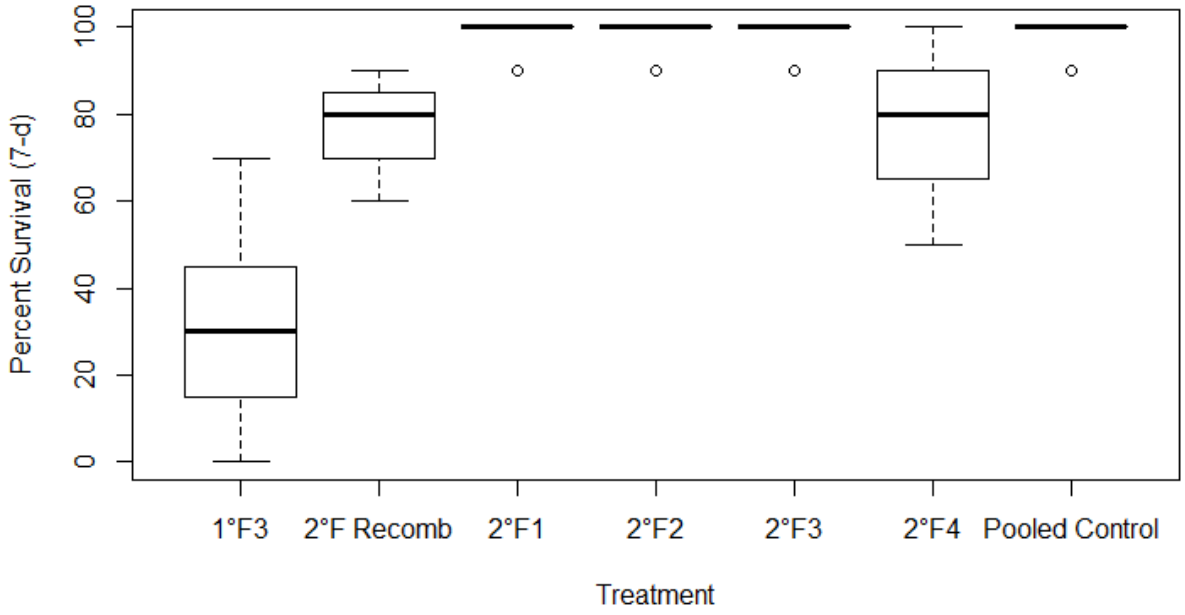


**Figure A-4:** Boxplot of OSPW Influenced GW – 1 *H. azteca* mean survival data (± standard deviation) for primary and secondary fraction treatments.